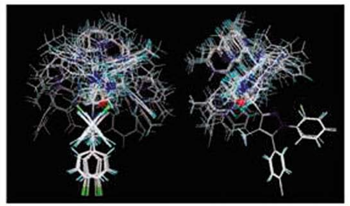
The Cannabinoid Receptors

Edited by

Patricia H. Reggio



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The Receptors

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ISBN: 978-1-58829-712-9 e-ISBN: 978-1-59745-503-9
DOI 10.1007/978-1-59745-503-9

Library of Congress Control Number: 2008939120

Printed on acid-free paper

springer.com

Preface

The identification of the cannabinoid CB1 receptor as the mediator of short-term and a mediator of long-term retrograde inhibition of synaptic transmission has changed the cannabinoid field profoundly. For with this discovery, the CB1 receptor moved from a G-protein-coupled receptor (GPCR) associated predominantly with the drug abuse field, into the neuroscience mainstream. Compared with other neurotransmitter systems, the endocannabinoid system is quite unique. The endogenous cannabinoid ligands, N-arachidonoylethanolamine (anandamide) and sn-2-arachidonoylglycerol (2-AG) are not small cationic ligands stored in vesicles, but rather are lipophilic ligands synthesized on demand from the lipid bilayer itself. Previously discovered ligands of the CB1/CB2 receptors, including those derived from cannabis, share the characteristic of high lipophilicity with these endogenous cannabinoids.

The CB1 receptor has been shown to have a high level of ligand-independent activation (i.e., constitutive activity) in transfected cell lines, as well as in cells that naturally express the CB1 receptor. This property likely is essential for the receptor to maintain a *cannabinoid tone* in the central nervous system (CNS). This property also permits the receptor to be modulated not only by agonists, but also by inverse agonists. The CB2 receptor, found predominantly in the immune system, also exhibits high levels of constitutive activity and inverse agonists for CB2 have been identified.

The cannabinoid receptors CB1 and CB2 each couple to intracellular G proteins (predominantly via Gi/Go proteins) in order to transduce agonist binding into a cellular response. Signaling by the two receptors can differ markedly, as indeed can signal transduction through each individual receptor in response to various ligands. The divergence of signaling is regulated at various stages – from G-protein coupling to activation of effectors, and in many cases appears to be cell-type specific. The intracellular domains important for G-protein coupling differ between each receptor subtype, and differential G-protein activation by agonists has been characterized. Mutation studies of CB1/CB2 have identified regions important for ligand binding, activation, and desensitization. Receptor modeling studies combined with mutation studies have been able to identify the molecular toggle switch for

CB1 activation and have led to molecular design criteria, for example, for the production of neutral antagonists.

Over the centuries, the plant for which the cannabinoid receptor was named, *Cannabis sativa L.*, has been used for a myriad of medicinal purposes, as well as for its psychotomimetic effects. Studies with CB1 knockout mice have shown that the CB1 receptor is primarily responsible for mediating the effects of the psychoactive principal in cannabis, Δ^9-THC. Physiological and behavioral analysis of CB1-knockout mice has provided important new insights into CB1 receptor function in mammals, which include roles in learning and memory, analgesia, appetite regulation, neuroprotection, as well as endocannabinoid-mediated retrograde signaling at synapses. Today, cannabinoid agonists have been suggested to have potential therapeutic uses such as appetite stimulants, analgesics, antiemetics, antidiarrheals, antispasmodics, tumor antiproliferative agents, antiglaucoma agents, and as agents for the treatment of diseases associated with inappropriate retention of aversive memories such as post-traumatic stress disorders and phobias. Cannabinoid CB1 antagonists/inverse agonists have been suggested to have potential therapeutic uses as appetite suppressants and as agents that improve memory.

This book is designed to introduce newcomers to the cannabinoid field. It begins at the molecular level with cannabinoid ligand synthesis and structure–activity relationships; then moves to the molecular pharmacology of the cannabinoid receptors and the endocannabinoid system; and, culminates in the whole animal pharmacology and therapeutic applications for cannabinoid drugs. New putative cannabinoid receptors are also discussed here, as are challenges for future research. It is hoped that this book will serve as a useful guidebook to what continues to be a fascinating field.

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Part I
Cannabinoid Receptor Ligands and Structure–Activity Relationships

Structure–Activity Relationships of Classical Cannabinoids

Raj K. Razdan

Abstract In this chapter an overview of the more recent developments in the structure–activity relationships (SARs) of classical cannabinoids is discussed, especially the profound pharmacological effects produced by various chemical entities in the side chain at C-3, the hydroxyl at C-1, C-11, and hydroxyalkyl chains at C-6. Also cardiovascular studies point to the presence of a novel cannabinoid subtype receptor and the antagonist activity of cannabidiol has opened up new areas for research. Ligands, which had either a unique pharmacological profile, were potent agonists, partial agonists/antagonists, or were CB2 selective, were identified, generating leads with the potential to be drugs in the treatment of various diseases.

Keywords Classical cannabinoids · Tetrahydrocannabinols · Structure activity relationships · Endocannabinoid system · Cannabinoid receptors · Vanilloid receptors · Pharmacological activity · Cannabinol · Cannabidiol · Cardiovascular activity

1 Introduction

It is well known [1–5] that cannabinoid research developed from the study of the pharmacological effects of the plant material from marijuana (*Cannabis sativa*). Earlier work by Adams and Todd had shown on the basis of degradation studies and ultraviolet (UV) that the natural material had a basic tricyclic benzopyran structure with a double bond either at the Δ^9- or Δ^8-position in the alicyclic ring (Fig. 1). They also showed that analogs with a double bond in the 6a,10a-position are not found in the plant, are UVactive, and had a pharmacological profile similar to the natural (–)-Δ^9-Tetrahydrocannabinol (THC) isolated from the plant. Adams and Loewe, using the dog-ataxia test, carried out extensive SAR studies in the series and established that the biological activity varied with the

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_1,

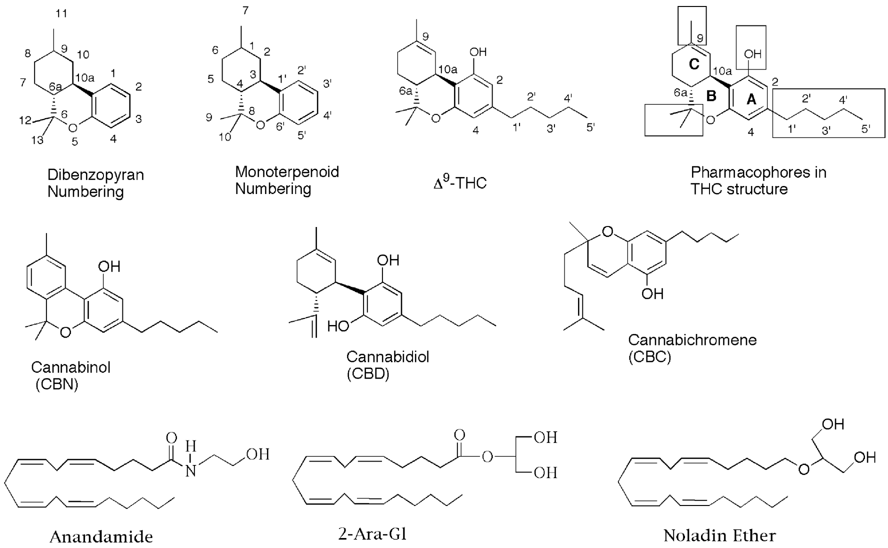


Fig. 1 Tetrahydrocannabinol numbering system, structures of selected natural products, and endocannabinoid system

position of the alkyl substituent in the side chain at C-3. The most potent compound in the series was found to be the 1′,2′-dimethylheptyl-pyran (DMHP) derivative which was about 500 times more potent than synhexyl, an analog with a n-hexyl side chain. They also showed that the natural THC (Δ^9-THC) was several-fold more active than the synthetic analog synhexyl. This work led to the development of various heterocyclic analogs of DMHP and resulted in potent agonists like SP-1, BRL 4664, etc. (Fig. 2). In 1964, the

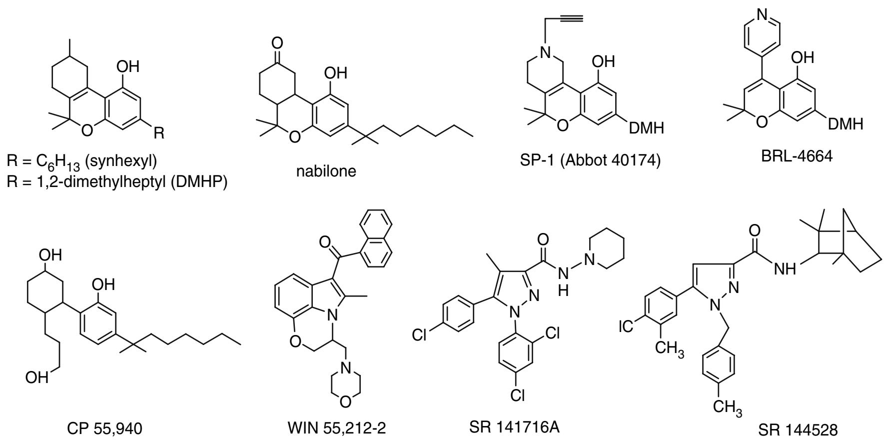


Fig. 2 Structures of selected cannabinoids

elegant work of Gaoni and Mechoulam [6] established, on the basis of nuclear magnetic resonance (NMR), that the position of the double bond in the alicyclic ring was in the Δ^9-position. It was thus determined that the active constituent of the plant, Δ^9-THC, is an ABC-tricyclic ring system having a benzopyran moiety (Fig. 1). At this time several analogs were synthesized and some were isolated from the plant, for example, Cannabinol (CBN) which had the same template as Δ^9-THC. Extensive SARs were developed in the series and these compounds were designated as *Classical Cannabinoids*. Hence, this class includes the natural product (–)-Δ^9-THC, the more stable and nearly equiactive isomer (–)-Δ^8-THC, other active constituents of the plant such as the CBN analogs mentioned above, cannabidiol (CBD) etc., as well as their synthetic analogs especially the $\Delta^{6a,10a}$ analogs developed by Adams and Todd. Although several synthetic strategies have been developed for the synthesis of analogs and metabolites in the Δ^9-THC series [7], most of the SAR studies in the cannabinoid field in the past several years have been carried out in the Δ^8-THC series, dictated mainly by ease of synthesis and the fact that the pharmacological profile of Δ^8-THC is very similar to Δ^9-THC, both in potency and activity.

(–)-Δ^9-THC is a partial agonist and binds equally well to the two G-protein-coupled receptors, CB1 and CB2, discovered in mammalian tissue. CB1 occurs both inside and outside the central nervous system (CNS) and CB2 is found mainly in the periphery [8–10]. The initial SAR studies of classical cannabinoids had pointed to three pharmacophores in the template, the most important being a lipophilic side chain at C-3, the hydroxyl at C-1, and a hydroxyl group at C-11. As a result, several potent agonists were developed. At about the same time the work at Pfizer resulted in the development of nonclassical cannabinoids, such as (–)-CP 55,940 and (–)-CP 55,244, which pointed toward the additional fourth pharmacophore, the southern aliphatic hydroxyl (SAH), and led to the discovery of the CB1 receptor. With this background most of the structural modifications for SAR studies [4, 5, 10–12] were carried out in the pharmacophores mentioned above. The importance of these sites has been borne out by molecular modeling studies [13–16] and is well accepted in the field.

In this article, an overview of the more recent developments in the SAR of classical cannabinoids is presented. These developments have resulted in several ligands which are either potent CB1 agonists, partial agonists/antagonists, or CB2 selective agonists.

2 SAR Studies

2.1 Aliphatic Side Chain at C-3

In recent years extensive SAR studies were carried out on modification of the aliphatic side chain including the effect of chain length and its substitution by methyl groups, substitution by various groups such as halogen, cyano, amido,

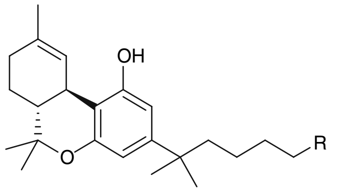
etc., at different carbons particularly the terminal carbon of the chain, the introduction of the rigid acetylene group/double bond at various positions in the side chain and other changes in the side chain.

2.1.1 Chain Length and Its Substitution by Methyl Groups

Cannabinoid activity is retained if the alkyl chain has minimum three to eight carbon atoms, the optimum being five to seven carbons, and is enhanced by the presence of a gem-dimethyl group at C-1′. Activity is also enhanced by the substitution of a 1′,2′-dimethylheptyl group. The former branching pattern is generally used and preferred, as the latter pattern introduces two chiral centers and leads to threo and erythro diastereomeric mixtures. A systematic study of the effect of a methyl substituent on each carbon of the n-pentyl side chain of Δ^9-THC indicates [17–19] that 1′- or 2′-methyl analogs are the most potent and there is relatively little difference between the R and S isomers in either set of compounds. A series of 1′,1′-dimethylalkyl-Δ^8-THC analogs with side chains of 2–12 carbon atoms was studied and showed that even the undecyl analog had significant affinity and was inactive in vivo. A quantitative SAR study of these analogs showed that, for optimum affinity and potency, the side chain must be of a length which will permit its terminus to loop back in proximity to the phenolic ring of the cannabinoid.

2.1.2 Substitution by Various Groups

The nature of the substituent has a profound effect on activity; the substitution of the terminal carbon of the n-pentyl chain in (–)-Δ^8-THCs by a halogen [20] such as a bromo, iodo, or a trifluoromethyl group increased the binding affinity and potency in the tetrad tests 2–40 times while the 5′-fluoro derivative was less active compared to (–)-Δ^8-THC. Similar modest effects in pharmacological profile were noted with the azido and amino substitutions [21]. However, a study of several cyano analogs of 1′,1′-gem-dimethyl-Δ^8-THC showed [22] that they had very high CB1-binding affinity (0.36–13 nM) and high in vivo potency as agonists. Two analogs, **1** and **2** (Fig. 3) had extremely high potency (ED_{50}, 0.0047 and 0.006 mg/kg for the tail-flick and spontaneous activity respectively) in the tetrad tests. The dimethylcarboxamido analog **3** also showed a similar profile of enhanced binding affinity and in vivo activity. In contrast the sulfonamido group can lead to compounds, as in **4**, with a unique profile, which have high binding affinity but are practically devoid of agonist effects. This provides a lead for the development of antagonists with a template different from Sanofi's pyrazole-based CB1 antagonist, SR 141716A. Furthermore, these side-chain derivatives have provided further insights in this cannabinoid pharmacophore. Traditionally, it has been assumed that a hydrophobic pocket accommodates the side chain but this study suggests that, in this region, the presence of a nitrile or a carboxamide group, which is polar but not negatively charged, enhances the interaction between the ligand and the receptor.



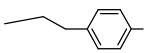
	R	CB1 (K_i, nM)	
1. (O-581)	-CN	0.36 ± 0.14	Potent agonist
2. (O-774)	$-CH_2CN$	0.6 ± 0.05	Potent agonist
3. (O-1125)	$-CON(CH_3)_2$	0.86 ± 0.06	Potent agonist
4. (O-606)	-CO-NH  $-SO_2NH_2$	29 ± 6	No agonist effects

Fig. 3 Effect of various substituents on the terminal carbon of the C-3 chain

2.1.3 Introduction of Regions of Planarity (Acetylene Groups) or Rigid Angles (Cis-Double Bonds) at Various Positions in the Side Chain [23–25]

Although it is well known that the flexible nature of the side chain plays a crucial role in the activation of the cannabinoid receptor, the precise nature of this interaction is not clear. A series of analogs with structurally restrained side chains of varying lengths were therefore studied in mice for their effect on binding affinity and potency. It was found that receptor affinity was the same for the acetylene and saturated side-chain analogs, whereas double bond substitution increased affinity 10-fold. Moreover, the relationship between affinity and potency in some of the acetylene derivatives was found to be 10-fold less than that of Δ^8-THC; however, this potency/affinity ratio was restored when the triple bond was changed to a cis-double bond. Additionally, an acetylene at C2′–C3′ in the octyl and nonyl side chains (**9** and **10**) showed antinociception selectivity by as much as 70-fold (Fig. 4). In contrast, several high-affinity acetylene derivatives, especially those with cyano substitutions at the terminus of the side chain (e.g., O-823, **5**) were partial agonists or were inactive. Some of these low-efficacy, high-affinity ligands, such as O-823, antagonized [25] the effects of cannabinoids in the guinea pig ileum. In a follow-up study they were examined in the GTPγS-binding assay and found to be devoid of agonist effects [26, 27]. These compounds were effective antagonists in that they blocked the agonist effects of several potent cannabinoids in this assay, but were not very effective in blocking the pharmacological effects of Δ^9-THC in vivo. Pretreatment with low doses of these compounds was without efficacy on the in vivo effects of THC in mice whereas high doses tended to increase rather than diminish the effects of Δ^9-THC. It appears that they have very weak agonist effects that mask their antagonist effects.

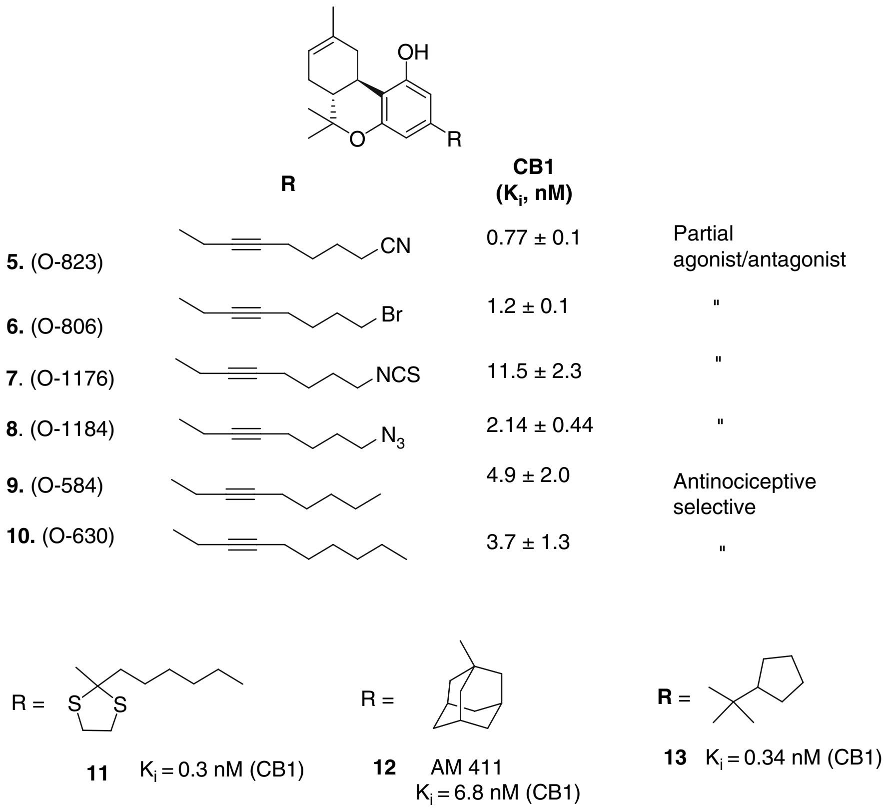


Fig. 4 Selected tetrahydrocannabinols with planar regions and larger groups in the side chain

These side-chain derivatives are the first compounds structurally related to THC that possess partial agonist/antagonist properties. At present an explanation for these unique effects is lacking. The pharmacological selectivity exhibited by some analogs may be explained by multiple transduction pathways for the CB1 receptors. This is supported by recent [28, 29] suggestions of the coupling of CB1receptors to both Gs and Gi/o proteins. However, it is possible that these agonists are interacting with as-yet-unidentified receptors. They seem unlikely to be CB2 receptors as their presence in the brain is questionable and CB2-selective analogs are not active in the tetrad tests.

2.1.4 Other Changes in the Side Chain

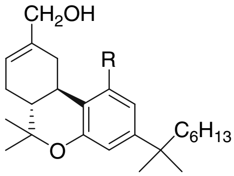
The substitution of the 2′-carbon by a cyclopentyl group with 1′,1′-gem-dimethyl group in the side chain (**13**, Fig. 4) retained [30] very high affinity for both CB1 ($K_i = 0.34$ nM) and CB2 ($K_i = 0.39$ nM) receptors. Even the cycloheptyl analog

retained high affinity to both CB1 ($K_i = 0.94$ nM) and CB2 ($K_i = 0.22$ nM) receptors. It was also found that the 1′,1′-dimethyl substitution in THCs can be replaced by cyclic moieties such as dithiolane, dioxolane, cyclopenty,l etc., with retention of potent affinity to both CB1 and CB2 receptors. In vitro pharmacological testing found the dithiolane analog **11** to be a potent CB1 agonist ($K_i = 0.32$ nM) [31]. Similarly, substitution of an 1-adamantyl group in place of the n-pentyl side chain of Δ^8-THC provided [32] a potent and efficacious CB1 agonist AM411 (**12**). The activity of these THC analogs suggests the presence of a quite large subsite within the binding pocket of CB1 and CB2 receptors.

2.2 The Hydroxyl at C-1

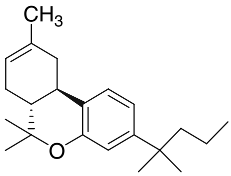
From traditional cannabinoid SAR it was known that the presence of a phenolic hydroxyl is very important for eliciting CB1 affinity, and its substitution by a methoxy group, hydrogen or fluorine atom, decreased both CB1- and CB2-binding affinities with marked effects on CB1. Recent work [33–35] has shown (Fig. 5) that even 1-deoxy or 1-methoxy-THCs, appropriately substituted at C-3 and C-11 can retain potent activity at both CB1 and CB2 receptors, and the 1-methoxy-THCs show more CB2 selectivity. SAR studies have indicated that either eliminating the hydroxyl group at C-1 (1-deoxy analogs) or changing it to a methoxy and at the same time decreasing the length of the DMH side chain at C-3 (1′,1′-dimethylbutyl, DMB, being optimal, e.g., **16**) enhances CB2 selectivity, which can be affected by the presence of a hydroxymethyl or an exo-cyclic group at C-9. In a very recent report [36] on the activity of 2′R- and 2′S- 1-methoxy and 1-deoxy-3-(2′-methylalkyl)- Δ^8-THCs with alkyl side chains of three to seven carbon atoms, it was found that all these compounds had greater affinity for the CB2 than the CB1 receptor. Some of them had good affinity for CB2 ($K_i = 13$–47 nM) and little for CB1 ($K_i = 1493$ to $>10{,}000$ nM) receptors. Also in the 1-deoxy series, the 2′S-methyl compounds generally showed greater affinity for the CB2 receptor than the corresponding 2′R isomer (see discussion of CB2 selectivity below).

From SAR studies has emerged the development of water-soluble cannabinoids, an area of growing importance and interest. Since cannabinoids are generally very lipid-soluble, solubilizing agents for pharmacological studies are used, but these agents, which have pharmacological effects of their own, can be avoided by making available cannabinoids which are water-soluble. By the formation of various esters of phenols, which hydrolyze at different rates, a series of water-soluble cannabinoids [37, 38] was developed in the early 1970s. A similar approach, and applying it to the recently developed potent THC agonists, led to the development of O-1057 (**20**, Fig. 6). It is a potent agonist [39, 40] and has affinity for both CB1 ($K_i = 8.36$ nM) and CB2 ($K_i = 7.95$ nM) receptors. It inhibits forskolin-stimulated cyclic AMP



14. R = OH (HU-210)

15. R = H JWH-051



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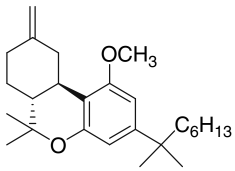
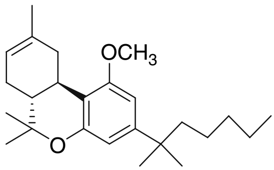
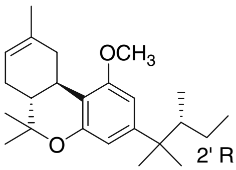


17.



18. JWH- 229



19. JWH- 359

Compound	CB1 (k_i, nM)	CB2 (k_i, nM)	CB1/CB2
14. R = OH (HU-210)	0.73 ± 0.11	0.52 ± 0.05	1.4
15. R = H JWH-051	1.2 ± 0.1	0.032 ± 0.019	37.5
16.	677 ± 132	3.4 ± 1.0	199
17.	> 20,000	19 ± 4	1052
18. JWH- 229	3134 ± 110	18 ± 2	174
19. JWH- 359	2918 ± 450	13 ± 0.2	224

Fig. 5 Selected tetrahydrocannabinols with CB2 selectivity

production by both CB1- and CB2-transfected CHO cells and has a potency similar to that of CP 55,940 and exceeding that of Δ^9-THC, especially as an analgesic ($ED_{50} = 0.02$ mg/kg, i.v.) and was antagonized by SR 141716A. At present, the potential of O-1057 for clinical application as an analgesic is under investigation.

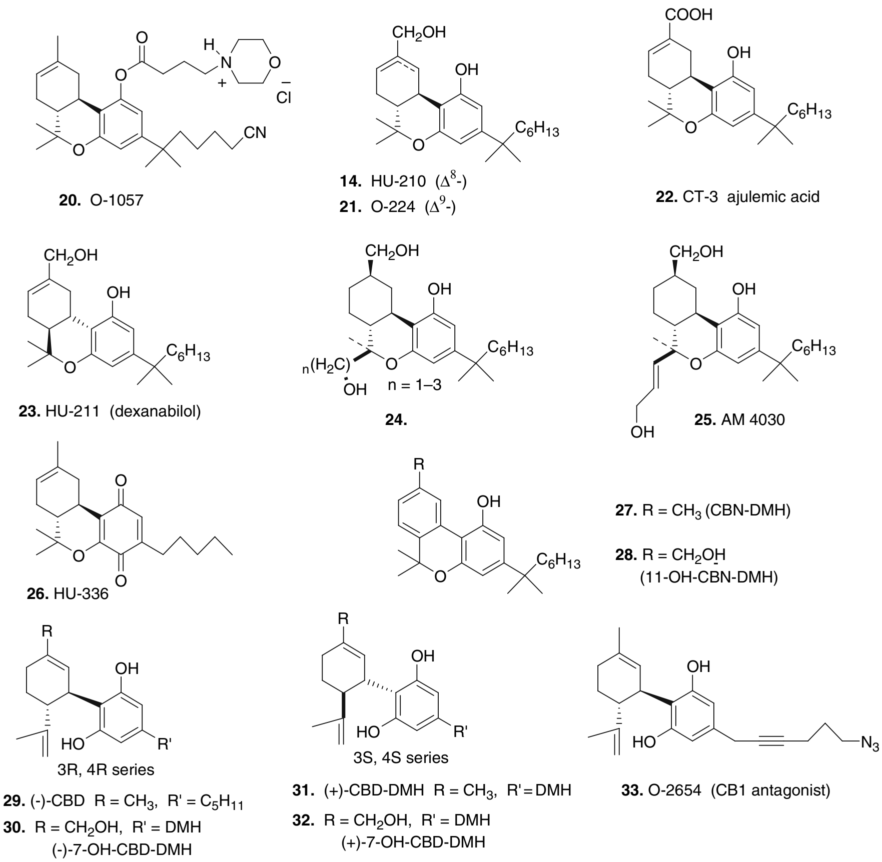


Fig. 6 Selected examples of CB1 ligands

2.3 The C-11 Position in the Alicyclic Ring

SAR studies [41–43] show that the presence of a hydroxyl group at C-11 is not a prerequisite for activity since the 9-nor compound retains activity. However, the metabolite of Δ^9-/Δ^8-THC, (i.e., 11-hydroxymethyl-THC) is approximately three times more active than the parent compound. With this background, the (–)-11-hydroxymethyl derivatives of both Δ^8- and Δ^9-THC-DMH (**14** and **21** respectively, Fig. 6) were synthesized and tested [44–49] for activity. They are (HU-210, **14** in the Δ^8- and O-224, **21** in the Δ^9-series) some of the most potent THCs known to be having very high CB1-binding affinities ($K_i = 0.7$ and 0.4 nM, respectively). It is interesting to note that the enantiomer of HU-210, (i.e., HU-211, dexanabilol **23**) is devoid of (–)-Δ^9-THC-like activity and is presently undergoing clinical development as a neuroprotective agent. This reinforces the importance of stereoselectivity in receptor–ligand interactions.

Even the C-9 aldehyde, also a metabolite, is quite potent ($K_i = 2$ nM) but the C-9 acid [50] has poor affinity ($K_i = 108$ nM) and is much less active. The lack of activity in the acid led to the development of CT-3 (ajulemic acid, **22**), which has shown analgesic/anti-inflammatory effects. It is presently under clinical development.

In general, the incorporation of a hydroxyl group at C-11 increases the binding affinity to CB1 receptors. The position of the double bond in the alicyclic ring has little effect on activity and is Δ^9- > Δ^8- > $\Delta^{6a,10a}$-. However, in the $\Delta^{7,8}$- isomers activity is retained when the 9-methyl group is *beta* but the *alpha*-isomer is much less active. When the double bond is eliminated by reduction, the hexahydro analogs are obtained, which have a 9α or 9β substitution. In this series [51, 52] the analogs retain high affinity and potency to both CB1 and CB2 receptors and it was found that the equatorial, 9β-methyl or hydroxymethyl, analogs are more potent than their axial (9α) counterparts. The activity is, however, adversely affected by substitution by a hydrogen or a fluorine atom at C-9. The presence of a ketone group, as in nabilone (Fig. 2) or a β-hydroxyl at C-9, retains activity. Nabilone is marketed as an agonist for the treatment of nausea in cancer therapy and as an appetite stimulant in AIDS patients.

2.4 Modifications at C-6 Position and the 1:4 Quinones in Ring A of Classical Cannabinoids

2.4.1 Modifications at C-6 Position

The SAR studies in the $\Delta^{6a,10a}$-series by Adams' group had shown that optimum activity was obtained by the presence of a gem-dimethyl group at C-6. Further SAR studies in our laboratory showed [53] that high activity and potency was retained in the (equatorial) 12β-hydroxy-Δ^8-THC. With this background Tius and Makriyannis' groups [12, 54, 55] developed hybrid cannabinoids, which incorporate the structural features of Δ^9-THC and the nonclassical cannabinoid CP 55,940. The binding affinity showed that the equatorial β-hydroxypropyl analog (**24**) had higher affinity than the α-axial epimer at C-6. Further analogs in the series were examined, which had restricted rotation at this site and resulted in very potent analogs such as AM 4030 (**25**, Fig. 6) with high binding affinity to CB1 ($K_i = 0.7$ nM) and CB2 ($K_i = 8.6$ nM).

2.4.2 1:4 Quinones in Ring A

In 1968, Mechoulam's group had reported [56] the formation of a hydroxyquinone from the oxidation of CBD, which cyclized to the corresponding Δ^8-THC under acid conditions. The structure of the CBN quinone derivative has now been confirmed by X-ray crystallography [57]. These 1:4 quinones in the Δ^8-THC (i.e., **26**), CBD and CBN series displayed antiproliferative activity in several human

cancer cell lines in vitro and the CBD analog significantly reduced cancer growth of HT-29 cancer in nude mice. It is interesting to note that these 1:4 quinones do not bind to CB1 receptors and the mechanism of their anticancer activity is unclear.

In summary, it is important to emphasize that classical cannabinoids, in general, bind to both CB1 and CB2 receptors and, as discussed above, it is clear that modifications at the hydroxyl at C-1 and at C-9 result in ligands with CB2 selectivity. An excellent discussion of CB2 selective ligands is reported in a recent review by John Huffman [58]. From a study of 1-methoxy- and 1-deoxy-Δ^8-THCs the following SAR conclusions were drawn: (1) the presence of a 1′,1′-dimethyl-alkyl side chain enhances both CB1- and CB2-binding affinities. However, the length of the chain has more effect on CB1 than on CB2. This is particularly more pronounced in the 1-deoxy-Δ^8-THC series where ligands with very short side chains retain good CB2 selectivity. (2) Introduction of an 11-hydroxy group enhances affinity for both receptors but the enhancement is more for CB1 affinity compared to CB2, and as a result CB2 selectivity is lowered. (3) In general the 1-methoxy analogs show lower binding affinities to both CB1 and CB2 receptors compared to their 1-deoxy counterparts.

The most CB2-selective compound found in the series [36] was JWH-359 (**19**, Fig. 5; $K_i = 2918$ nM for CB1 and 13 nM for CB2; CB1/CB2 = 224) and the compound with the highest affinity to CB2 was JWH-051 (**15**, $K_i = 0.032$ nM for CB2) but it has low selectivity (CB1/CB2 = 37.5) [33].

3 Other Cannabinoids Found in the Plant

It is well documented that numerous other cannabinoids are present in the plant but only a few such as CBN, CBD, and cannabichromene (CBC) have been studied for their biological activity. Limited SAR studies have been carried out in the CBN and CBD series and the conclusions are discussed below.

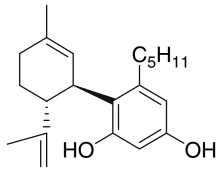
3.1 Cannabinol (CBN)

It is one of the first cannabinoids to be synthesized, which demonstrated the basic skeleton of the THC structure. Interest in CBN analogs increased after it was reported [59] that the affinity of CBN to CB2 receptors was greater than that of Δ^9-THC. The SAR studies were carried out in hopes of getting novel CB2-selective agonists with a CBN template, but none of the analogs showed high CB2 selectivity although some analogs showed high binding affinities to both CB1 and CB2 receptors [60, 61]. The SAR indicate the following: (1) There are differences in the binding profiles of THCs and CBN analogs, and the removal of the phenolic hydroxyl decreases CB1-binding affinity much more in the CBN series than in the THC series. Thus in the 3-(1′,1′-dimethylheptyl) analogs (e.g., **27**) there is a 400-fold decrease in the CBN series versus a 30-fold

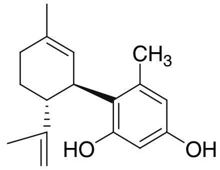
decrease in the THC series. (2) In the 1-deoxy-CBN series, when a hydroxyl group is present at C-11, the side chain length has relatively little influence on the selectivity (CB1/CB2 ratio) in contrast to the finding in the THC series. (3) CB1/CB2 selectivity is reduced if the planarity of ring C is increased as in CBN analogs. (4) High CB2-binding affinity was found only when the phenolic hydroxyl was present (e.g., **28**). The only exception was in the 1-deoxy-CBN series when the hydroxyl was at C-11. Thus the presence of a hydroxyl group either at ring C or ring A enhances binding affinity to the CB2 receptor.

3.2 (–)-Cannabidiol (CBD, 29)

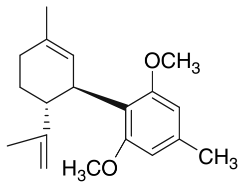
It is one of the major constituents of the plant and does not possess any of the psychotropic effects of Δ^9-THC but has several pharmacological effects which have been confirmed in vitro assays and in animal and human tests. CBD does not bind to CB1 or CB2 receptors and even its DMH analogs [62] bind very weakly to both receptors. Thus (–)-CBD-DMH binds to the CB1 receptors with a K_i above 10 μM and to the CB2 receptors with a K_i of 1800 nM. However, much higher affinities to both CB1 and CB2 were found in the enantiomer (+)-CBD-DMH (**31**) ($K_i = 17.4$ and 211 nM, respectively). A similar pattern was observed in the 7-hydroxy series, (+)-7-OH-CBD-DMH (**32**) bound with a K_i of 2.5 nM to CB1 and 44 nM to CB2, while the values were 4400 and 671 nM, respectively, in the (–)-enantiomer (3R, 4R series, **30**). Similarly the metabolites with an acid group at C-7 showed differences in the (+) – and (–) – series. It can be concluded that in CBDs, higher binding affinity to CB1 and CB2 is found in the 3S, 4S series compared to the natural 3R, 4R series. However, it should be noted that not all cannabinoid activities are CB1/CB2-mediated. The pharmacological interaction of CBD and its analogs with vanilloid receptors, their activity for cellular uptake, and their enzymatic hydrolysis of anandamide were reported [63] recently. There is now a great deal of interest in CBD for its therapeutic potential, for example, in the management of epilepsy, as an anti-inflammatory agent, as a neuroprotective antioxidant, etc. Pertwee's group has recently shown [64] that CBD can antagonize the cannabinoid agonists R-(+)-WIN 55,212 and CP 55,940 in the mouse isolated vas deferens, and CBD shares this ability with the CB1 antagonist SR 141716A. It was also found that CBD produces this antagonism at concentrations well below those at which it binds to CB1 receptors and antagonizes α_1-adrenoceptor agonists insurmountably. An SAR study of various CBD analogs showed [65] that O-2654 (**33**, Fig. 6), an analog in which the 4′-pentyl group of CBD was replaced by a 6″-azido-2″-hexyne side chain, was as potent as CBD in producing surmountable antagonism of R-(+)-WIN 55,212 in vasa deferentia. However, this antagonism was produced with a potency ($K_B = 85.7$ nM) which was similar to its CB1-binding affinity (114 nM) suggesting that it is a competitive CB1 receptor antagonist.



34. abnormal-CBD



35. O-1602
agonist



36. O-1918
antagonist

Fig. 7 Selected ligands with cardiovascular activity

This is in contrast to that of CBD. Furthermore it appears that O-2654 may be a neutral CB1 receptor antagonist. This is an interesting finding as it provides a potential template for the development of silent antagonists of CB1 receptors.

Cardiovascular activity is another area where CBD is involved. Cardiovascular studies of cannabinoids by Kunos and co-workers [66–69] has led to the postulation of an endothelial site, distinct from CB1 or CB2 receptors, that contributes to anandamide-induced vasodilation in the mesenteric circulation and possibly elsewhere. The non-CB1 endothelial receptor is coupled to Gi/Go, and abnormal-CBD (**34**, Fig. 7), which does not bind to CB1 receptors, is an agonist and CBD is an antagonist. SAR studies in the two series resulted in the development of O-1602 (**35**) as a more potent agonist and O-1918 (**36**) as a more potent antagonist. Both of them have a methyl group in the benzene ring in place of the n-pentyl side chain, as longer chains decreased activity. The role of cannabinoids in the regulation of blood pressure is most interesting and there is a distinct possibility that these studies will result in ligands, useful in the treatment of cardiovascular disease.

3.3 Cannabichromene (CBC)

CBC is also found in significant quantities in the plant. Except that it is an antimicrobial agent [70] very little is known about its pharmacological effects and no significant SAR studies have been reported in recent years.

4 Concluding Remarks

Much progress has been reported in recent years on SAR studies and on our understanding of the interaction of classical cannabinoids with the cannabinoid CB1 and CB2 receptors. Several important pharmacophore areas have been defined and this should help in the future design of selective ligands and the possible discovery of novel cannabinoid subtype receptors. With the identification

of the *endocannabinoid system*, the acceptance of cannabinoids in medical treatment is assured. From this field one is hopeful that several new drugs will soon become available.

Acknowledgments We thank the National Institute of Drug Abuse, Grants DA-05488, DA-08904, and DA-09789 for financial support.

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Endocannabinoids and Their Synthetic Analogs

V. Kiran Vemuri and Alexandros Makriyannis

Abstract The discovery of endogenous cannabinoids has provided a basis for understanding the structural requirements for activation of the two known cannabinoid receptors CB1 and CB2. The endocannabinoids are fatty acid analogs represented by *N*-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG). These endogenous ligands are biosynthesized and deactivated by a number of enzymes and a transporter system, all of which can be modulated by ligands whose structures can encompass the essential endocannabinoid features. For this reason a significant amount of work has sought to develop synthetic ligands structurally related to AEA and 2-AG. These ligands are being used to explore the structural features of the different cannabinergic proteins and have also proven to be valuable pharmacological tools for studying the physiology and biochemistry of the endocannabinoid system.

Keywords Endocannabinoid · Anandamide · AEA · 2-Arachidonoylglycerol · 2-AG · Cannabinoid receptors · Structure–activity relationship · Retroanandamide · FAAH inhibitors · AEA transport inhibitors

1 Introduction

The endocannabinoid system (ECS) is represented by CB1 and CB2, two well-characterized G protein-coupled receptors [1–4]. Furthermore, there is evidence for the presence of additional cannabinoid receptors [5–8]; however, their full characterizations are still incomplete. Additionally, the ECS encompasses a family of membrane phospholipid-derived endogenous ligands known as endocannabinoids, and a number of functional proteins involved in the synthesis, transport, and inactivation of these endogenous ligands [9–11]. Through their

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_2,

interactions with cannabinoid receptors (CBRs), endocannabinoids regulate cellular levels of cyclic adenosine monophosphate (cAMP) through a G protein-mediated interaction with adenylyl cyclase [12, 13] and also modulate the functional properties of voltage-gated ion channels including Ca^{2+} channels, Na^{+} channels, various types of K^{+} channels, and ligand-gated ion channels such as 5-HT_3, nicotinic acetylcholine, and glycine receptors [14]. For example, it has been shown that activation of the CB1 receptor inhibits voltage-gated Ca^{2+} channels (L, N, and P/Q type) [15–17] and activates the inward rectifying K^{+} channels [17, 18]. Conversely, the CB2 receptor has not been shown to be coupled to ion channels [19, 20]. Other cannabinoid receptor signaling pathways have also been described, such as the activation of mitogen-activated protein kinase (MAPK), regulation of the phosphatidylinosityl-3-kinase, and signaling through ceramide as a second messenger [21].

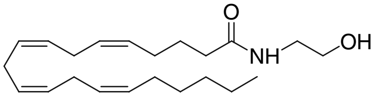
The therapeutic opportunities available through modulation of the ECS are very diverse and include CNS neuro-inflammation [22, 23], drug addiction [24], alcoholism and neuropsychiatric disorders [25, 26], inflammatory and neuropathic pain [27, 28], autoimmune diabetes [29], multiple sclerosis [30, 31], stress and anxiety responses [32], nociceptive behavior [33], obesity [34, 35], and sexual behavior [36]. The ECS is also known to be involved in Alzheimer's disease [37], chronic liver disease [38], blood pressure regulation and cardiovascular disease [39], allergic asthma [40], inflammatory bowel disease and other gastrointestinal allergic disorders [41], and atherosclerosis [42].

During the past 15 years, a number of endogenous ligands associated with endocannabinoid function have been isolated and characterized. The most prominent of these are *N*-archidonoylethanolamine (anandamide, AEA, **1**, Fig. 1) [43] and 2-arachidonoylglycerol (2-AG, **5**, Fig. 1) [44], both of which are capable of activating the CB1 and CB2 receptors. In this review, we describe the best-studied endocannabinoid or endocannabinoid-like compounds and describe several representative synthetic endocannabinoid analogs possessing interesting biochemical properties, many of which are being used as pharmacological probes. We also outline the pharmacophoric requirements for the various endocannabinoid targets.

2 Endocannabinoids and Related Ligands

Several fatty acid derivatives have been identified and characterized as endogenous ligands for the cannabinoid system (Figs. 1–8). These endocannabinoids are phospholipid-derived eicosanoid ligands, many of which are agonists capable of activating both CB1 and CB2 receptors through a $G_{i/o}$ mechanism [45]. A characteristic feature of these molecules is that they are biosynthesized from cell membrane components and are produced locally on demand [11].

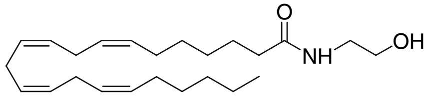
Anandamide (AEA, **1**, Fig. 1), the ethanolamine amide of arachidonic acid, was the first endocannabinoid isolated from water-insoluble fractions of



1

Anandamide (AEA)

Ki = 32 nM (CB1)
= 1930 nM (CB2)



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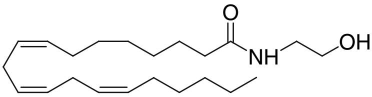
N-Docosatetraenoylethanolamine

Ki = 34 nM (CB1)



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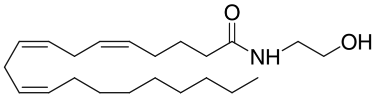
N-Dihomo-γ-linolenoylethanolamine

Ki = 54 nM (hCB1)



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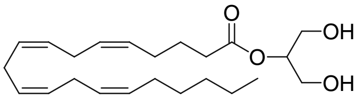
Meadethanolamide

Ki = 753 nM (CB1)
= 1810 nM (CB2)



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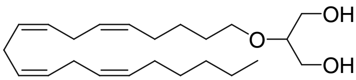
2-Arachidonoylglycerol (2-AG)

Ki = 472 nM (CB1)
= 1400 nM (CB2)



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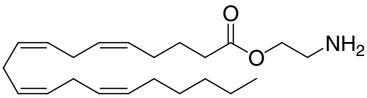
2-Arachidonyl glyceryl ether (Noladin ether)

Ki = 21 nM (CB1)
= 3000 nM (CB2)



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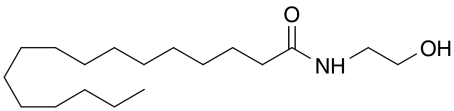
Virodhamine

EC_{50} = 1906 nM (CB1)
= 1401 nm (CB2)

Fig. 1 Endogenous cannabinoids



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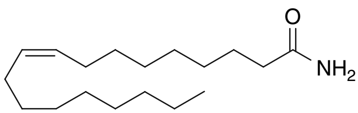
N-Palmitoylethanolamine (PEA)

Ki = 23.8 μM (CB1)
= 13.9 μM (CB2)



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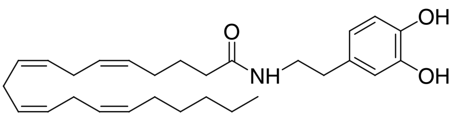
Oleamide (ODA)

Ki = 1140 nM (CB1)

Fig. 2 Representative endocannabinoid-related *N*-acylethanolamines and amides



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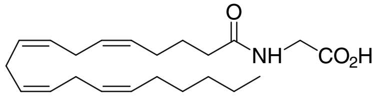
N-Arachidonoyldopamine

Ki = 0.25 μM (CB1)
= 12 μM (CB2)



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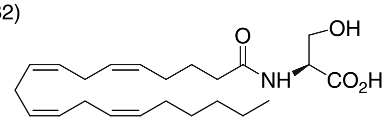
N-Arachidonoylglycine



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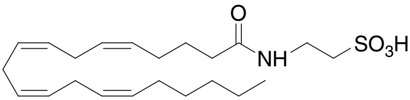
N-Arachidonoyl-L-serine

Fig. 3 Representative endogenous arachidonamides



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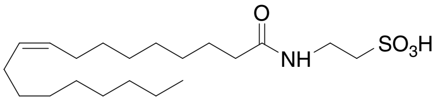
N-Arachidonyltaurine



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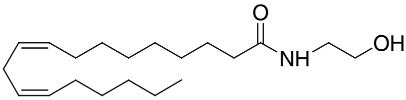
N-Oleoyltaurine

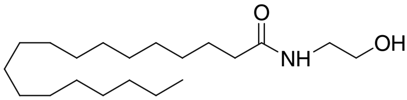
Fig. 4 *N*-Acyl taurines



15

N-Linoleoylethanolamine (LEA)

Ki = 10 μM (CB1)
= 25 μM (CB2)



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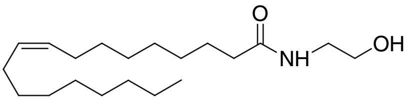
N-Stearoylethanolamine (SEA)
Ki = >1000 nM (CB1)



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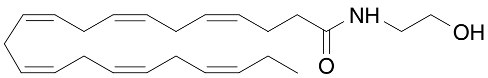
N-Oleoylethanolamine (OEA)

Ki = 20 μM (CB1)
= 31 μM (CB2)

Fig. 5 *N*-Acyl ethanolamines of C-18 fatty acids



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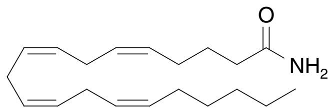
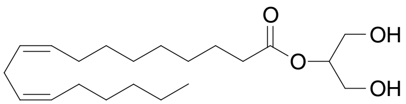
N-Docosahexaenoylethanolamine (DHEA)
Ki = 324 nM (CB1)



19

Arachidonamide

Fig. 6 Representative endogenous polyunsaturated *N*-acylethanolamines and amides



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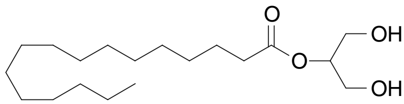
2-Linoleoylglycerol



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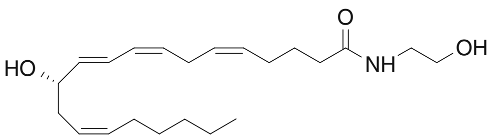
2-Palmitoylglycerol

Fig. 7 Representative endocannabinoid-related long chain fatty acid glycerol esters



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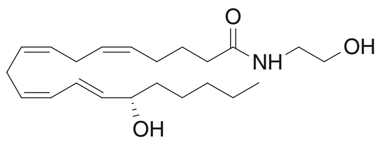
(12*S*)-HAEA
Ki = 150 nM (CB1)



23

(15*S*)-HAEA
Ki = 600 nM (CB1)

Fig. 8 Lipoxygenase metabolites of AEA

porcine brain [43]. It is biosynthesized from the membrane phospholipid precursor, *N*-arachidonoylphosphatidylethanolamine (NAPE), which is formed by the transfer of an arachidonoyl (AA) group from phosphatidylcholine (PC) to phosphatidylethanolamine (PE) through the catalytic action of Ca^{2+}-dependent *N*-acyltransferase (NAT). NAPE is then cleaved by a NAPE-specific phospholipase-D (PLD) to generate anandamide [46]. The NAPE-PLD enzyme was recently cloned and is classified as a member of the zinc metallohydrolase family of the β-lactamase fold having no homology with the known phospholipase-D family [47, 48]. A more recently identified biosynthetic pathway for AEA involves the hydrolysis of NAPE by a phospholipase C enzyme. The resulting phospho-AEA is then dephosphorylated to AEA by a tyrosine phosphatase [49]. AEA is an endogenous cannabinoid neurotransmitter that binds to the CB1 receptor (Ki 32 nM), has a lower affinity for CB2 (Ki 1930 nM), and was shown to be a

cannabinoid-partial agonist [15, 50]. It modulates cAMP levels [51], inhibits N-type calcium currents through a pertussis toxin-sensitive pathway [16], inhibits the electrically evoked twitch-response of the mouse *vas deferens* (MVD) [52], and produces a series of behavioral responses in mice that are characteristic of cannabinoids, such as antinociception, hypothermia, hypomotility, and catalepsy [53]. A distinguishing feature of AEA is that it undergoes facile hydrolysis by fatty acid amide hydrolase (FAAH) [54], an enzyme found in the same regions of the brain in which the CB1 receptor is present [55]. This enzyme was isolated from mammalian brains and cloned [56, 57], and its structure determined by X-ray crystallography [58]. During receptor-binding assays, AEA degradation can be prevented by the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) [59]. Over the past decade, accumulated evidence has suggested the existence of a high-affinity carrier-mediated transport of AEA in neurons and astrocytes. This anandamide transport (ANT) system appears to play a key role in terminating the biological actions of AEA by the reuptake of extracellular AEA into the cells followed by FAAH-mediated intracellular hydrolysis [60, 61]. Other endocannabinoid ethanolamides include *N*-docosatetraenoylethanolamine (**2**, Fig. 1) and *N*-dihomo-γ-linolenoylethanolamine (**3**, Fig. 1) which bind to the CB1 receptor with Ki values of 34 and 54 nM, respectively [62], and inhibit both forskolin-stimulated adenylyl cyclase as well as the MVD twitch response [63]. Meadethanolamide (**4**, Fig. 1) inhibits forskolin-stimulated adenylyl cyclase activity with potency similar to AEA in CHO-CB1 cells. In L cells expressing the human CB1 receptor, it exhibits a Ki of 753 nM, while in AtT-20 cells expressing the human CB2 receptor, it binds with a Ki value of 1810 nM [20, 64].

2-Arachidonoylglycerol (2-AG, **5**, Fig. 1), an endocannabinoid first isolated from canine gut [44], was shown to be another endogenous cannabinoid and is present in the brain at concentrations approximately 170-fold higher than AEA [65]. 2-AG is a monoglyceride which binds to both the CB1 and CB2 receptors with Ki values of 472 and 1400 nM, respectively [44]. It acts as a full agonist [66] and produces the characteristic effects of Δ^9-THC such as antinociception, immobility, immunomodulation, and inhibition of electrically evoked contractions of the MVD [44]. 2-AG is formed within the cell membrane mainly by two different biosynthetic pathways involving hydrolysis of inositol phospholipids and 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine [67–70]. The biological actions of 2-AG are known to be terminated by hydrolytic cleavage through a monoglyceride lipase (MGL), a serine hydrolase. MGL has been shown to be localized in brain areas rich in CB1 receptors and has been cloned by homology from a rat brain cDNA library [71]. Both AEA and 2-AG are produced on demand in response to elevations of intracellular calcium [72], and act as autocrine and/or paracrine mediators near the sites of their biosynthesis [73].

2-Arachidonyl glyceryl ether (noladin ether,**6**, Fig. 1) was reported to be an endogenous cannabinoid isolated from porcine brain [74]. In mice it produces catalepsy, decreases ambulation, hypothermia, antinociception, and reduces intestinal motility [74]. This compound was shown to stimulate NG108-15 cells, inducing rapid transient elevation of the intracellular free Ca^{2+} concentrations

through a CB1 receptor-dependent mechanism [75]. Recently, noladin ether was shown to act as a full agonist at the CB2 receptor with an efficacy equivalent to CP55,940 and 2-AG as shown by the concentration-dependent increase in [^{35}S]GTPγS binding in CB2-CHO membranes as well as the concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity in whole CHO cells expressing CB2 receptors and in HL-60 cells, and these effects are reversed by the CB2 antagonist AM630 [76, 77]. However, the endogenous nature of noladin ether has been disputed [78].

Virodhamine (**7**, Fig. 1), another endogenous cannabinoid, was shown to be present in peripheral tissues at concentrations much higher than AEA, and at lower concentrations than AEA in the brain [79]. In vivo, it acts as a full agonist at the CB2 receptor and as a partial agonist/antagonist at the CB1 receptor when tested for hypothermia in mice [79]. Virodhamine was also shown to act as a weak anandamide uptake inhibitor (IC$_{50}$ 123 μM) [79].

Palmitoylethanolamine (PEA, **8**, Fig. 2), a potent analgesic lipid, was originally detected in high levels in the brain, liver, and muscle tissues of the rat and guinea pig [80]. It behaves as an agonist, and binds to peripheral CB2-like cannabinoid receptors on mast cells, which are bone marrow-derived cells of the nervous system capable of playing an important role in tissue inflammation and neuroimmune interaction [81]. PEA was shown to inhibit the forskolin-stimulated accumulation of cAMP in BV-2 cells, a response prevented by pertussis toxin and not antagonized by either SR141716A or SR144528. It produces many of the nonpsychoactive effects of cannabinoids, an observation which led to the hypothesis that PEA acts through G$_{i/o}$ protein-coupled receptors that are pharmacologically distinct from CB1 and CB2 receptors [82, 83].

Oleamide (*cis*-9-octadecenamide, ODA, **9**, Fig. 2), is known to be a sleep-inducing agent and was first identified in the cerebrospinal fluid of sleep-deprived cats [84]. ODA and AEA are known to produce similar pharmacological effects in mice [85]. ODA is also a substrate for FAAH [86] and shares other characteristics with AEA, which include inhibiting the binding of the nonselective CBR agonist [^{3}H]CP55,940 and that of the selective CB1 antagonist [^{3}H]SR141716A, enhancing [^{35}S]GTPγS binding, and inhibiting forskolin-stimulated cAMP accumulation in mouse neuroblastoma N1E-115 cells [87].

3 The Endocannabinoid Metabolome

The field of endocannabinoid lipidomics has claimed the existence of a more-extended list of fatty acid ethanolamides, amides, glycerol esters, as well as newly identified *N*-acyl dopamines and *N*-acyl aminoacids, all possessing a wide diversity of biological functions. It was also observed that many of these molecules behave as congeners which potentiate the binding of the known endocannabinoids to the CBRs. Although they may not seem to have significant intrinsic affinity toward the CBRs, they produce effects related to endocannabinoid physiology and many of

these compounds are substrates for endocannabinoid-metabolizing enzymes. This extended family of fatty acid lipid conjugates appears to be produced in a combinatorial fashion and constitutes a broad spectrum of endocannabinoid- and endocannabinoid-related ligands. The physiological roles of these compounds remain to be fully explored and are the focus of intensive research efforts. It is tempting to speculate that these new families of endogenous ligands have pleiotropic actions acting on a number of lipid-related targets including endocannabinoid and perhaps vanilloid proteins. One intriguing potential role for at least some of these lipid conjugates is that of serving as carriers for central neurotransmitters across the blood–brain barrier. Presumably, these conjugates are subsequently metabolized in the brain to release the individual bioactive ligands such as dopamine or the CNS-active amino acids. Below we outline some of the lipid conjugates that have received recent attention.

N-Arachidonoyldopamine (NADA, **10**, Fig. 3) is an endocannabinoid detected in rat brain, with the highest concentrations in the striatum, hippocampus, and cerebellum [88]. NADA was shown to inhibit the binding of [^{3}H]SR141716A to CB1 (Ki 0.25 μM) and acts as a potent inhibitor in the MCF-7 cancer cell proliferation assay. NADA induces hypothermia, hypolocomotion, catalepsy, and analgesia in the mice tetrad test, as well as a rapid transient increase in intracellular free calcium concentrations in N18TG2 cells expressing CB1, thus behaving as an agonist with a potency superior to AEA [89]. NADA was also shown to activate the vanilloid VR1 receptor and is postulated to act as an endovanilloid [88].

N-Arachidonoylglycine (NAG, **11**, Fig. 3) is formed by the condensation of glycine with arachidonoyl-CoA mediated by the enzyme ACGNAT (acyl-CoA:glycine *N*-acyltransferase) [90, 91]. NAG was isolated from bovine and rat brains along with *N*-arachidonoylalanine and *N*-arachidonoyl-GABA [92], and was shown to be a potent FAAH inhibitor [93]. However, *N*-arachidonoylserine (**12**, Fig. 3), also detected in bovine brain, had no affinity for the CB receptor, and in vivo results indicate that it is an endogenous ligand for the abnormal cannabidiol (Abn-CBD) receptor [42].

Recently, a new class of aminoacyl endocannabinoids, the *N*-acyl taurines (Fig. 4), whose production is regulated by FAAH, were identified in vivo [94, 95]. These have been recognized as a distinct class of substrates for FAAH [96] and are found along with other *N*-acylethanolamines (NAEs) such as AEA (**1**, Fig. 1), PEA (**8**, Fig. 2), and OEA (**17**, Fig. 5) and primary amides such as ODA (**9**, Fig. 2) and arachidonamide (**19**, Fig. 6). The biological functions of the NATs, as well as their relevance to endocannabinoid signaling, remain to be determined.

Another C-18 unsaturated NAE congener structurally related to the endocannabinoids is *N*-linoleoylethanolamine (LEA, **15**, Fig. 5), which has been detected in mouse peritoneal macrophages [97]. LEA has a low affinity for the CB1 and CB2 receptors compared to AEA, exhibiting Ki values of 10 and 25 μM, respectively [98], and is involved in a cell signaling process, though not through the CBRs [99]. This compound produced catalepsy in mice with an ED_{50} of 37.5 mg/kg [100]. LEA and its hydroxylated analog have been reported

to be natural inhibitors of human brain FAAH [101]. *N*-Stearoylethanolamine (SEA, **16**, Fig. 5) was detected in rat, mouse, and human brains at levels higher than AEA. Although SEA exhibits low affinity for CBRs, it behaves as a cannabimimetic ligand in the mouse tetrad tests with a different time course and potency when compared to AEA [102]. Binding assays and kinetic experiments in the CB1 knock-out mice suggest that the regional distribution of the binding sites for SEA are different than the CB1 receptors, and it was proposed that the SEA binding may not be coupled to the $G_{i/o}$ proteins. SEA exhibits no significant stimulation of [^{35}S]GTPγS binding and has no effect on adenylyl cyclase, but potentiates the effect of AEA on forskolin-induced cAMP concentrations [102]. It has been recently proposed that LEA, SEA, and OEA act as vanilloid receptor agonists [103].

N-Docosahexaenoylethanolamine (DHEA, **18**, Fig. 6) has been detected in bovine retina along with 2-docosahexaenoylglycerol. Both compounds behave as weak CB1 agonists [15, 104, 105]. However, DHEA was shown to have a higher K^+ channel inhibiting potency compared to AEA [106] thereby suggesting the existence of signaling processes for eicosanoids that do not involve the CB receptors.

2-Linoleoylglycerol (**20**, Fig. 7) and 2-palmitoylglycerol (**21**, Fig. 7) were isolated along with 2-AG from mouse spleen [107]. Both esters do not bind to the CBRs and do not inhibit adenylyl cyclase. However, they independently or jointly potentiate the binding of 2-AG and its capacity to inhibit adenylyl cyclase and its effects in the tetrad behavioral tests on mice [107].

The amide and ester endocannabinoids are deactivated through a hydrolytic process involving FAAH and MGL, respectively. Additionally, endocannabinoids are metabolized through oxidative reactions mediated by various lipoxygenases and cyclooxygenase-2, which lead to ethanolamide analogs of hydroxy-eicosatetraenoic acids and the prostaglandins. Two such metabolites (12*S*)-HAEA (**22**, Fig. 8) and (15*S*)-HAEA (**23**, Fig. 8) have been identified, with (12*S*)-HAEA exhibiting affinities for both CB1 and CB2 which are nearly that of AEA, while (15*S*)-HAEA had a significantly lower affinity [108, 109]. Conversely, their order of potency is reversed when measuring inhibition of the electrically evoked contraction of MVD [110]. Both of these oxygenated AEA derivatives were found to be good FAAH inhibitors [111].

4 Structure–Activity Relationships of Some Synthetic Analogs of Endocannabinoids

Over the past decade a number of analogs were designed and synthesized based on systematic variations of the AEA and 2-AG structures. Many of these have proven to be very useful probes for the CBRs, endocannabinoid-deactivating enzymes, and the transport system. AEA and 2-AG analogs have also served to delineate the pharmacophoric requirements for CBR function as well as the

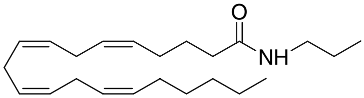
structural requirements of ligands as substrates or inhibitors for the endocannabinoid-deactivating proteins. In general, all arachidonic acid-based endocannabinoid analogs appear to favor the CB1 receptor over CB2. We summarize the most salient features of their structure–activity relationships (SARs) below.

4.1 AEA Head Group Modifications

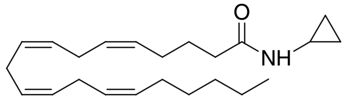
The optimal site for the hydroxyl group in AEA analogs is two carbons away from the amide group with the affinity and in vivo potency dropping drastically if the chain is extended beyond a propyl group. However, the presence of a hydroxyl group is not essential for optimal CB1 affinity, as arachidonamides with *N*-propyl (**24**, Fig. 9), *N*-cyclopropyl (**25**, Fig. 9), *N*-allyl (**26**, Fig. 9), *N*-propargyl (**27**, Fig. 9), and *N*-*t*-butyl groups exhibit an enhanced affinity for CB1 and are also more resistant to FAAH hydrolysis [98, 112, 113]. A similar trend is observed when the hydroxyl group is replaced by a chloro (**28**, Fig. 9) or a fluoro (**29**, Fig. 9) group. Ring structures such as the oxazoline and their methylated analogs and other bulkier aromatic systems attached directly to the amide nitrogen appear to reduce the affinity of ligands for CB1. Recently, it was reported that oxyhomologation of the amide bond to give *N*-hydroxylated analogs (**30**, Fig. 9) of AEA leads to ligands with enhanced affinities for the CB2 receptor [114].

4.2 (R)-Methanandamide and Related Analogs

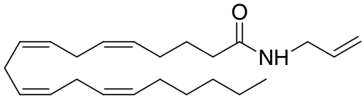
The rationale for synthesizing this class of compounds was to overcome their facile enzymatic hydrolysis both in vitro and in vivo while maintaining a high affinity for the CB1 receptor. It was shown that methyl substitution at the C-1′ and C-2′ positions of the *N*-hydroxyethyl group in AEA led to enhanced-metabolic stability toward FAAH. The best known of the methylated isomers (**31–34**, Fig. 10), (*R*)-methanandamide (AM356, **31**, Fig. 10) has a higher affinity for CB1 than AEA along with enhanced metabolic stability. Its higher potency was also demonstrated in vivo in the tetrad tests of hypothermia, hypokinesia, ring immobility, and antinociception in mice. AM356 is presently being used as a standard in cannabinoid pharmacology [115]. Substrate stereo-selectivity was seen with FAAH, as the *S*-isomer (**32**, Fig. 10) of AM356 is a better substrate than its *R*-enantiomer [59, 116]. Introduction of bulkier substituents neighboring the amido group in general provides compounds that are resistant to enzymatic hydrolysis [116]. Additionally, the presence of the hydroxyl group did not seem to be an essential requirement for FAAH hydrolysis of **35** and **36** (Fig. 10). It was observed, however, that the presence of the amide–NH hydrogen is required in FAAH substrates as tertiary amides



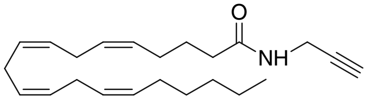
24
N-Propylarachidonamide
Ki = 7.3 nM (CB1)



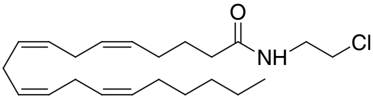
25
N-Arachidonoylcyclopropylamine (ACPA)
Ki = 2.2 nM (CB1)
= 715 nM (CB2)



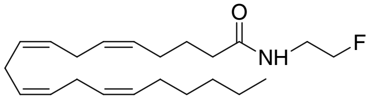
26
AM883
Ki = 9.9 nM (CB1)
= 226 nM (CB2)



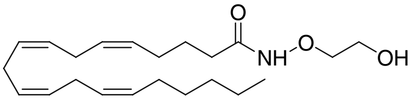
27
Ki = 10.8 nM (CB1)
= 290 nM (CB2)



28
AM881
Ki = 5.3 nM (CB1)
= 95 nM (CB2)



29
Fluoroanandamide
Ki = 8.6 nM (CB1)

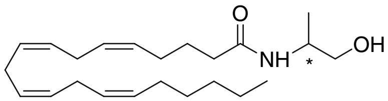


30
Ki = 470 nM (CB1)
= 81 nM (CB2)

Fig. 9 Synthetic AEA analogs

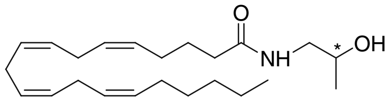
were totally resistant to hydrolysis. With the exception of the bishydroxyethyl amide (**37**, Fig. 10) [117], tertiary amides and substitutions involving bulkier groups at the C-1′ and C-2′ positions of the polar head group reduce affinity for the CB receptor [118].

Monomethylation or gem-dimethylation at the C-2 position of the AEA chain leads to compounds with improved CB1 affinities, while the presence of the hydroxyl group is not required (Fig. 11) [119–121]. A study of dimethylated AEA stereoisomers found the (C-2*R*,C-1′*R*)-dimethyl analog AM1116 (**38**, Fig. 11) to have the highest affinity for CB1 and metabolic stability to FAAH [121]. Conversely, the corresponding ethyl and isopropyl analogs are not tolerated well by the receptor [118, 122]. Calculations based on conformational

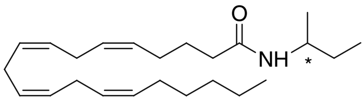


Methanandamide

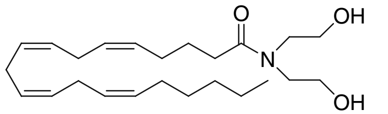
(*R*)-**31** (AM356) Ki = 17.9 nM (CB1)
= 868 nM (CB2)
(*S*)-**32** Ki = 173 nM (CB1)



(*R*)-**33** Ki = 119 nM (CB1)
(*S*)-**34** Ki = 26 nM (CB1)



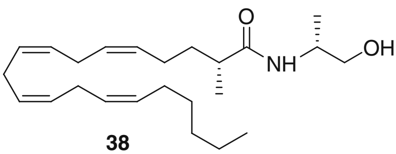
(*R*)-**35** Ki = 73 nM (CB1)
(*S*)-**36** Ki = 287 nM (CB1)



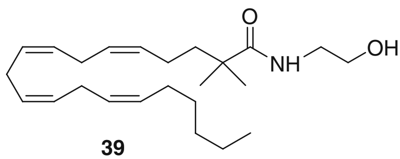
37
Ki = 174 nM (CB1)

Fig. 10 Synthetic AEA analogs that are resistant to FAAH hydrolysis

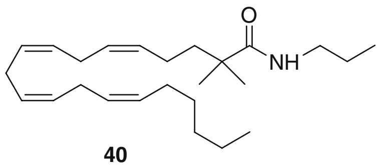
memories method coupled with ligand-receptor docking used AM1116 (**38**, Fig. 11) and its diastereomers to refine the active state model of the CB1 receptor. Based on the computational data, it was postulated that anandamide and its congeners adopt a tightly curved U- (hairpin) or a J-shaped conformation at the CB1 receptor, and that transmembrane helices 2, 3, and 7 constitute the binding region for these ligands [123]. Additionally, in a multilamellar model membrane bilayer system, AEA adopts an extended conformation [124] with access to helices 3 and 6, both of which are believed to be involved in CB1 receptor activation [125].



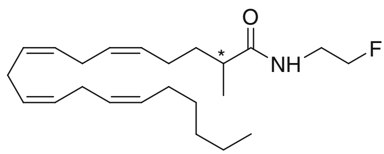
38
AM1116
Ki = 7.4 nM (CB1)



39
Ki = 7.4 nM (CB1)

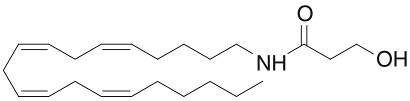


40
Ki = 6.9 nM (CB1)



(*R*)-**41** Ki = 22.4 nM (CB1)
(*S*)-**42** Ki = 15.3 nM (CB1)

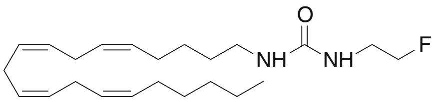
Fig. 11 High affinity methylated AEA analogs



43

AM1174

Ki = 114 nM (CB1)
= 3540 nM (CB2)



44

Ki = 55 nM (CB1)

Fig. 12 Representative retroanandamide and urea analogs of AEA

4.3 *Retroanandamides and Related Head Group Modifications*

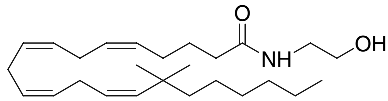
An important contribution to the medicinal chemistry of anandamides is the observation that when the position of the amide nitrogen and carbonyl groups are interchanged, the resulting compounds are significantly more stable to enzymatic hydrolysis. This new class of ligands is referred to as retroanandamides (AM1174, **43**, Fig. 12) [98]. Similarly, incorporating a urea functionality in the anandamide head group produces ligands which retain affinity for CB1 while being resistant to FAAH action [126]. Recently, it was shown that a one carbon homologation of anandamides leads to compounds that behave as partial agonists for both the CB receptors and are also stable to hydrolysis by FAAH [127].

4.4 *Tail Group Modifications of AEA*

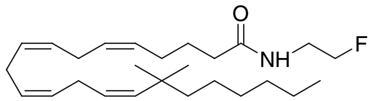
The SAR of the terminal 5 carbon chain of anandamide exhibits many similarities with the side chain moiety of the classical cannabinoids. It was thus shown that an increase in chain length up to an optimal seven carbon atoms, the introduction of methyl or gem-dimethyl substituents, as well as the addition of cyano or halogen groups at the terminal carbon lead to analogs with enhanced affinities for CB1 (Fig. 13). It was also observed that all these analogs share common pharmacological properties with Δ^9-THC as exemplified in the mouse tetrad tests and twitch inhibition in MVD [128–130]. The increase in CB1 affinity can be further enhanced by introducing optimal head group substituents as in fluoroanandamide **46** (Fig. 13) and methanandamide **49** (Fig. 13).

4.5 *Varying Degrees of Unsaturation*

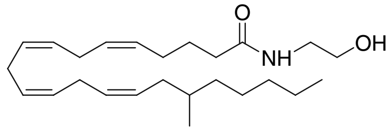
It has been observed that the optimal number of double bonds in the lipid backbone is four and that the affinity for the CB1 receptor drastically drops when the number is less than three. This SAR is reflected in the binding affinities



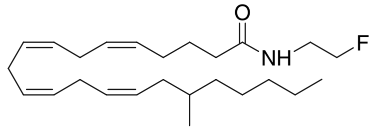
45
Ki = 7 nM (CB1)



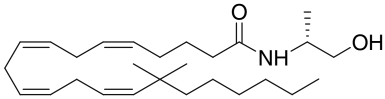
46
Ki = 1 nM (CB1)



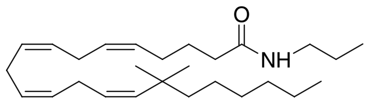
47
Ki = 11 nM (CB1)



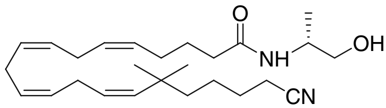
48
Ki = 3.3 nM (CB1)



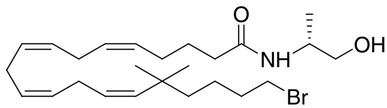
49
Ki = 1.7 nM (CB1)



50
Ki = 1.5 nM (CB1)



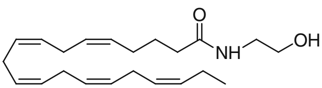
51
O-1812
Ki = 4.6 nM (CB1)



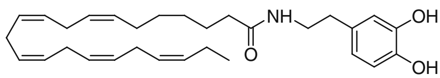
52
O-1860
Ki = 1 nM (CB1)

Fig. 13 Representative AEA analogs with modification of the terminal tail

of the endogenous ligands with a varying number of double bonds including PEA (**8**, Fig. 2), SEA (**16**, Fig. 5), OEA (**17**, Fig. 5), LEA (**15**, Fig. 5), *N*-dihomo-γ-linolenoyl- ethanolamine (**3**, Fig. 1), and meadethanolamide (**4**, Fig. 1). However, the CB1 receptor appears to tolerate the addition of two additional carbons as in *N*-docosatetraenoylethanolamine (**2**, Fig. 1) and the presence of one to two additional double bonds as in DHEA (**18**, Fig. 6), 5,8,11,14, 17-eicosapentaenoic acid ethanolamide (**53**, Fig. 14) [119, 122], and the head group analog *N*-docosapentaenoyl(ω-3)dopamine (**54**, Fig. 14), which acts as a weak AEA uptake inhibitor [89].

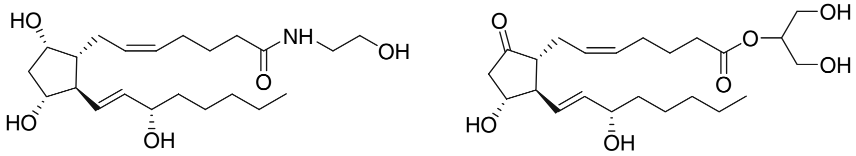


53
Ki = 162 nM (CB1)
5,8,11,14,17-Eicosapentaenoic acid ethanolamide



54
Ki = 700 nM (CB1)
N-Docosapentaenoyl(ω-3)dopamine

Fig. 14 Representative pentaenoic analogs of AEA



55
Prostaglandin-$F_{2\alpha}$-ethanolamide

56
Prostaglandin-E_2-glycerol ester

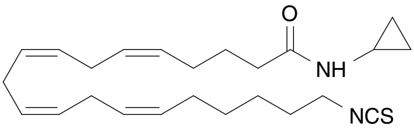
Fig. 15 COX-2 metabolites of AEA and 2-AG

4.6 Endocannabinoid COX-2 Metabolic Products

Prostamides and their analogous glycerol esters are biosynthesized from AEA [131] and 2-AG [132], respectively, through the COX-2 enzymatic pathway. An SAR study of some prostamide analogs with lipophilicities comparable to AEA showed that these compounds have very low affinities for the CB1 receptor when [^{3}H]CP55,940 was used as a radioligand [112, 133]. Prostamide $F_{2\alpha}$ (**55**, Fig. 15) [134] and the glycerol ester of PGE_2 (**56**, Fig. 15) [135] have been shown to modulate intracellular Ca^{2+}; however, these novel mediators may have distinct receptor-transduction pathways [136].

4.7 Covalent Probes

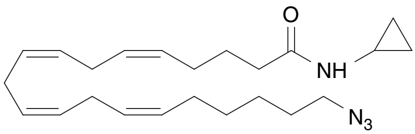
An approach for directly mapping key amino acid residues associated with the main binding domains of the CB receptors involves the use of covalent high-affinity probes [137]. The attachment site(s) of the ligand on the receptor are subsequently identified using proteomic approaches. Two types of such ligands based on the anandamide structure were developed. The first involves the introduction of the electrophilic isothiocyanato group, which reacts preferentially with cysteine residues at or near the CBR-binding site. The second type of ligand incorporates a photo-activatable azido moiety which forms a reactive species also capable of covalent attachment. A pair of such analogs in which the anandamide structure has been suitably optimized, AM3677 (**57**, Fig. 16) and AM3661 (**58**, Fig. 16), carrying the isothiocyanato and azido groups, respectively, at the terminal carbon were recently synthesized and shown to successfully label the CB1 receptor [138]. Work involving the identification of their respective site(s) of attachment to the receptor is currently being pursued.



57

AM3677

Ki = 1.3 nM (CB1)
= 48 nM (CB2)



58

AM3661

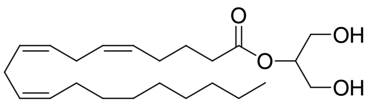
Ki = 0.9 nM (CB1)
= 57 nM (CB2)

Fig. 16 Covalent AEA probes for the CB1 receptor

4.8 2-AG Analogs

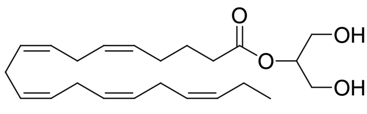
Unlike AEA, the number of reported analogs based on the 2-AG structure is relatively modest. Preliminary SAR for 2-AG analogs was reported based on their ability to induce rapid transient increases in intracellular free Ca^{2+} [105]. It was observed that 2-AG and its 1(3)-isomers had higher potencies than their ether-linked analogs. Compounds with three (**59**, Fig. 17) and five double bonds (**60**, Fig. 17) were more potent than their respective saturated analogs, and the presence of the double bond at the Δ^5 position of the C-20 lipid chain was considered essential for activity. It was also shown that the presence of a free hydroxyl group adjacent to the ester linkage was essential for agonist activity. Also, analogs in which the ester group is substituted by a keto group, as in ketone **61** (2-AGA109, Fig. 17), as well as its corresponding alcohol (2-AGA105) exhibited activities comparable to 2-AG [139].

The C-22 polyunsaturated fatty acid analogs such as 2-docosahexaenoyl (ω-3)glycerol failed to exhibit strong agonistic activity in the [^{35}S]GTPγS assay. However, the C-21 chain length analog with a terminal dimethylheptyl



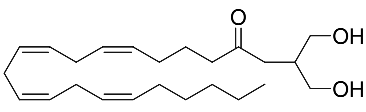
59

2-Eicosatrienoyl(ω-9)glycerol



60

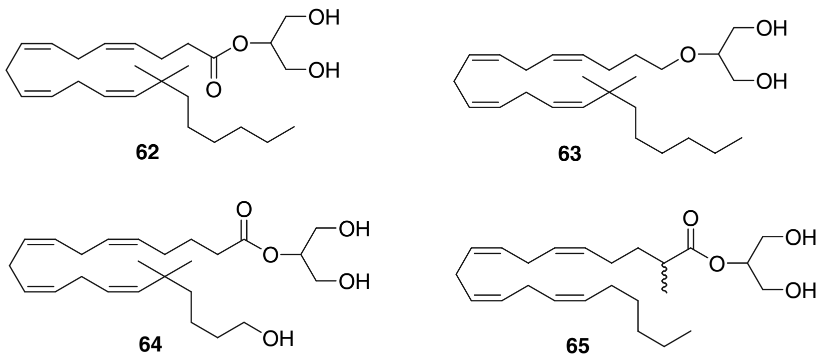
2-Eicosapentaenoyl(ω-3)glycerol



61

2-AGA109

Fig. 17 Structural analogs of 2-AG



62

63

64
O-2204
Ki = 3.2 μM (CB1)

65

Fig. 18 Methylated analogs of 2-AG

group **62** (Fig. 18) and its ether-linked analog **63** (Fig. 18) are agonists with enhanced efficacy [140]. Unlike AEA, the SAR of 2-AG analogs does not parallel that of the Δ^9-THC side chain. Thus, modified dimethylpentyl 2-AG analogs like O-2204 (**64**, Fig. 18) showed lower affinities for CB1 and were reported to act as weak FAAH and MGL inhibitors [141]. Also, the α-methylated derivative **65** (Fig. 18) was found to be more stable than 2-AG, and the metabolic stability of such analogs was found to be independent of their chirality [142].

4.9 Synthetic Modulators of Endocannabinoid Degradation and Transport

Endocannabinoid degradation assisted by transporter-mediated uptake is receiving a great deal of attention in cannabinoid pharmacology since these endocannabinoid proteins have been identified as useful targets for therapeutic intervention [143]. FAAH, a serine hydrolase, is the key enzyme responsible for the degradation of fatty acid amides such as ODA (**9**, Fig. 2) as well as ethanolamides such as AEA (**1**, Fig. 1) and OEA (**17**, Fig. 5) [56, 58, 84, 144]. The optimal pH for FAAH activity is near alkaline [145]. However, a second enzyme was recently reported to participate in the degradation of AEA at an optimal acidic pH of 5 and with a distinct substrate profile. For example, this enzyme is highly selective toward PEA (**8**, Fig. 2), a poor FAAH substrate, but is not inactivated by PMSF, a potent inhibitor of FAAH [146, 147]. Many of the known FAAH and anandamide transporter inhibitors are structurally related to the endocannabinoid ligands and will be briefly reviewed here.

4.9.1 FAAH Inhibitors

The early FAAH inhibitors include fatty acid trifluoromethylketones such as arachidonyltrifluoromethylketone (ATFMK, **66**, Fig. 19, IC_{50} 7.5 μM) [148] and oleyl TFMK (**67**, Fig. 19, IC_{50} 0.46 μM) [149, 150]. These ligands are believed to interact covalently with FAAH in a reversible manner. Another class of reversible covalent ligands includes fatty acid ketones substituted with electron-rich heterocyclic rings such as compounds **68** (Fig. 19) and **69** (Fig. 19). These heterocyclic substituents enhance the electrophilic character of the ketone group which serves as an electrophilic target for the FAAH serine hydroxyl group [151–153]. The first irreversible covalent FAAH inhibitor to be identified was phenylmethylsulfonyl fluoride (PMSF, IC_{50} 0.29 μM). This compound has been used in CB1 receptor-binding assays for FAAH-susceptible

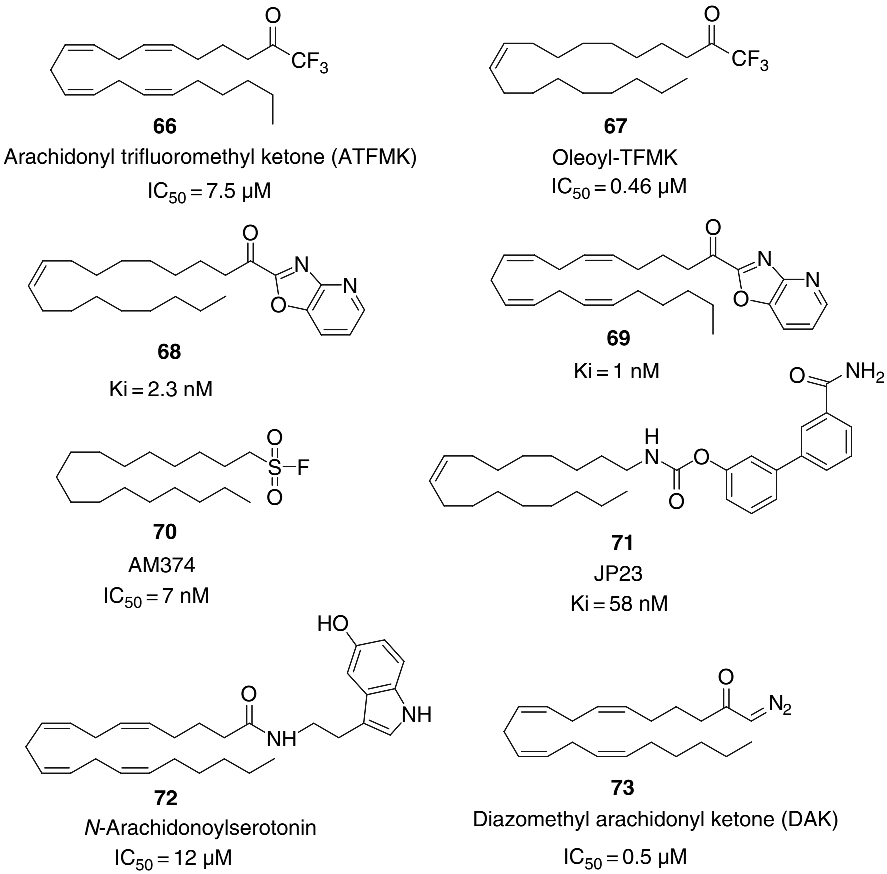


Fig. 19 Representative FAAH inhibitors

ligands such as the AEA analogs [54, 59]. More potent sulfonyl fluorides were later developed and are represented by the very effective and selective FAAH inhibitor AM374 (**70**, Fig. 19, IC_{50} 7 nM) [154]. Another class of irreversible FAAH inhibitors encompasses the carbamate functionality. Both sulfonyl fluorides and carbamates react with the same catalytic serine residue at the FAAH active site. The carbamate FAAH inhibitors include the oleyl analog JP23 (**71**, Fig. 19) [155] and URB597 (IC_{50} 4.6 nM) in which the arachidonyl group is substituted with the more compact biphenyl moiety [156]. Other FAAH inhibitors include *N*-arachidonoylserotonin (**72**, Fig. 19) [157] and diazomethyl arachidonyl ketone (DAK, **73**, Fig. 19, IC_{50} 0.5 μM) [158], which can also serve as a photo-activatable probe.

4.9.2 Anandamide Transport Inhibitors

Specific transporter systems for the reuptake of AEA [60] and 2-AG [159] have been described and partially characterized. The first reported successful AEA transport inhibitor is AM404 (*N*-(4-hydroxyphenyl)arachidonamide**, 74**, (Fig. 20) [60], while its meta-hydroxy isomer is significantly weaker [61]. The transporter system exhibits ligand stereoselectivity opposite that of the CB1 receptor with the (*S*)-methanandamide (**32**, Fig. 10) analog behaving as a superior inhibitor compared to its (*R*)-enantiomer (**31**, Fig. 10) [61]. In the AM404-related series, it has been observed that substrate recognition requires the presence of at least one cis double bond situated in the middle of the fatty acid chain. Olvanil (**75**, Fig. 20), OMDM 1 (**76**, Fig. 20), and OMDM 2 (**77**, Fig. 20), which belong to the oleyl series, maintain this pharmacophoric requirement and have been reported to be good transporter inhibitors [160, 161]. However, transport activity is optimal with substrates containing a minimum of four cis nonconjugated double bonds. AM1172 (**78**, Fig. 20) with a reverse carboxamide moiety is a potent transporter inhibitor. This retroanandamide ligand is very unique in design as it retains the key pharmacophoric features necessary for transport inhibition while maintaining metabolic stability against enzymatic hydrolysis [162]. A variety of novel transport inhibitors structurally related to AEA have been reported. A common feature of this group of ligands is the presence of a NH–amide bond in the head group attached to an aromatic or heteroaromatic ring as exemplified by molecules such as the indole analog VDM-13 (**79**, Fig. 20) [163], the 3-pyridyl analog (**80**, Fig. 20) [164], the furan compound UCM 707 (**81**, Fig. 20) [165], and arvanil (**82**, Fig. 20) [160], all of which have Ki values ranging between 0.8 and 10 μM. Although the SAR of AEA uptake inhibitors has received considerable attention and suggests the existence of a distinct target, the cloning of such a protein has not been accomplished yet. This raises the possibility of the existence of alternative mechanisms for the transport of these endocannabinoids across the cell membrane.

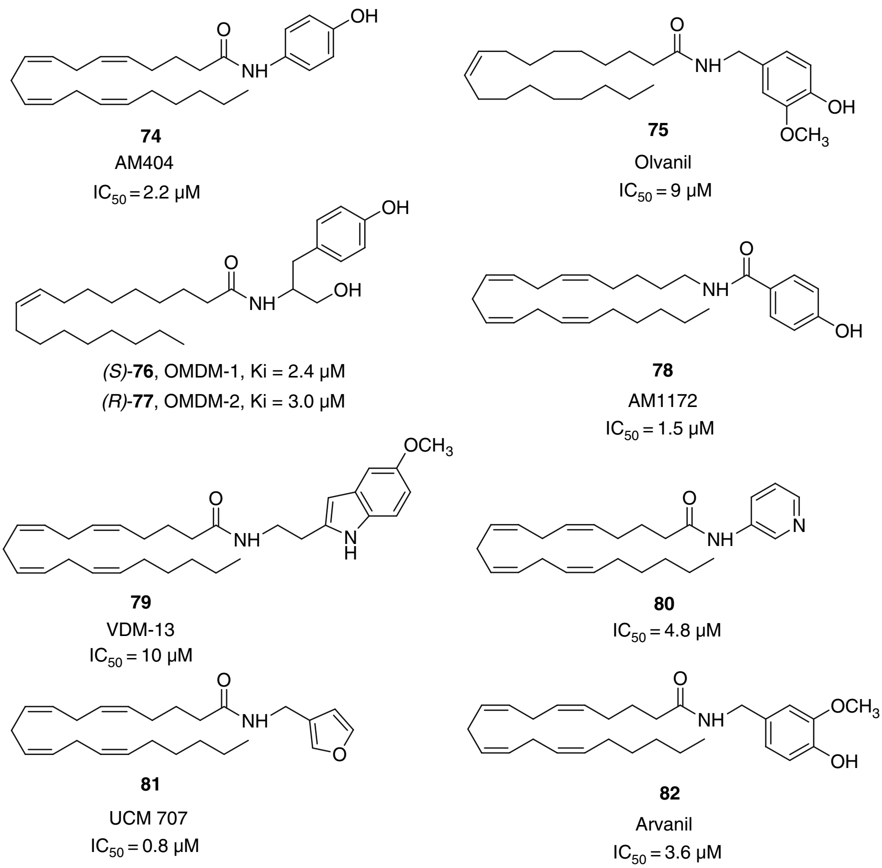


Fig. 20 Representative inhibitors of AEA transport

5 Conclusions

Significant medicinal chemistry efforts have sought to develop novel selective ligands for each of the key known endocannabinoid proteins. Many of these synthetic analogs are being used to explore in some detail the biochemical and physiological roles of the endocannabinoid system. An overview of these endocannabinoid-based lipid ligands is provided in this review. It has also become clear that the endocannabinoid system offers excellent opportunities for drug discovery, and there have been parallel efforts to develop structurally diverse compounds as potentially novel medications acting through the modulation of the cannabinergic functions. The design and development of these novel ligands make use of high-throughput screening or target-based approaches. These non-lipid *druggable* structures have not been discussed here, but can be found in other chapters within this anthology of cannabinoid research.

Acknowledgments This review is supported by grants from National Institute on Drug Abuse (DA03801, DA07215 and DA09158). We thank Drs. Richard I. Duclos, Jr. and Dai Lu for their discussions and technical help in preparing the manuscript.

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Cannabimimetic Indoles, Pyrroles, and Indenes: Structure–Activity Relationships and Receptor Interactions

John W. Huffman

Abstract A number of years ago it was found that 1-aminoalkyl-3-aroylindoles have affinity for the canabinoid receptor that is expressed in the central nervous system (CB1 receptor). More than 100 of these aminoalkylindoles were prepared and structure–activity relationships (SARs) were developed for these compounds. Subsequently it was found that the aminoalkyl substituent could be replaced by a straight chain alkyl group of four to six carbon atoms without loss of affinity for the CB1 receptor. One of these indoles, 1-propyl-3-(1-naphthoyl) indole was found to have relatively high affinity for the cannabinoid receptor that is expressed in the periphery (CB2 receptor), but with little affinity for the CB1 receptor. In order to explore the SAR for these cannabimimetic 3-(1-naphthoyl)alkylindoles a number of compounds have been synthesized, some of which have very high affinity for the CB1 receptor and others which are highly selective for the CB2 receptor.

On the basis of a suggested pharmacophore for the cannabimimetic indoles, a series of 1-alkyl-3-(1-naphthoyl)pyrroles was prepared, one of which had modest affinity for the CB1 receptor and was active in vivo. Subsequent work led to the development of a series of 1-alkyl-2-aryl-4-(1-naphthoyl)pyrroles, some of which have high affinity for the CB1 and/or CB2 receptor. Two groups have reported the synthesis of cannabimimetic indenes, which serve as rigid models for the CB1 receptor. Through a combination of molecular modeling and studies of mutant receptors a body of evidence has been acquired, which indicates that cannabimimetic indoles, and by extension pyrroles and indenes, interact with the CB1 and CB2 receptors primarily by aromatic stacking.

Keywords Cannabinoid · Aminoalkylindoles · CB1 receptor · CB2 receptor · Pyrrole · Indene · Indole

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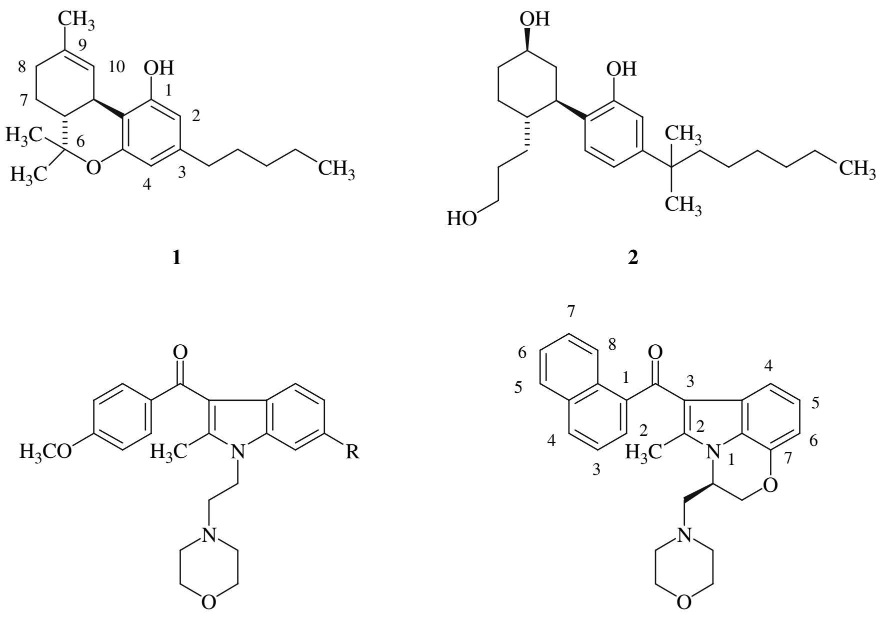
P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_3,

1 Introduction

Marijuana (*Cannabis sativa L.*) has been used as a recreational and ritual drug as well as a therapeutic agent, for many centuries, probably preceding the dawn of recorded history [1]. The structure of the principal psychoactive constituent of *Cannabis*, Δ^9-tetrahydrocannabinol (Δ^9-THC, **1**), was elucidated by Gaoni and Mechoulam in 1964 [2]. On the basis of the partially reduced dibenzopyran structure of THC a comprehensive set of structure–activity relationships (SARs) was developed for compounds structurally related to Δ^9-THC (Fig. 1) [3–5]. In the early 1980s a group at Pfizer developed a series of very potent nontraditional cannabinoids of which CP-55,940 (**2**) is a typical example [6–9]. These compounds lack the dibenzopyran ring present in traditional cannabinoids, but exhibit typical cannabinoid pharmacology and the SAR developed for the traditional dibenzopyran-based cannabinoids was extended to these compounds.

In 1991, workers at the Sterling Research Group reported that during the course of a program directed toward the development of nonsteroidal anti-inflammatory drugs they found that pravadoline (**3**) unexpectedly inhibited the



1 **2**

3 R = H
12 R = Br
13 R = I

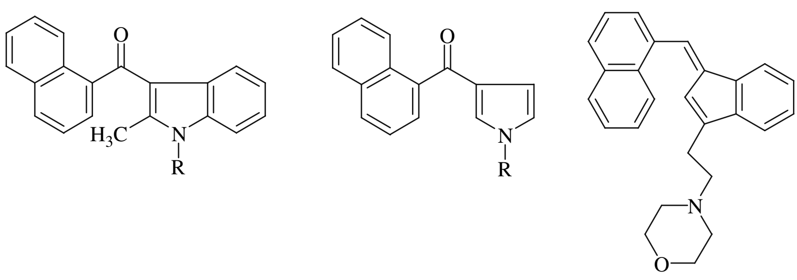
4

Fig. 1 Structures of Δ^9-THC, CP-55,940, pravadoline, and related compounds and WIN-55,212-2

contractions of the electrically stimulated mouse vas deferens (MVD) [10]. In addition to inhibiting contractions of the MVD, pravadoline and related compounds also inhibit adenylate cyclase and are antinociceptive in vivo. These compounds, which were described as aminoalkylindoles (AAIs) based upon the presence of an alkylamino group appended to the indole nitrogen, were found to interact with a G-protein-coupled receptor in the brain. It was suggested that this was the cannabinoid receptor and subsequent work confirmed that compounds of this group bind to the cannabinoid receptor found in the central nervous system (CNS), some with quite high affinity [11]. One of these compounds, WIN-55,212-2 (**4**), has very high affinity for both principal cannabinoid receptors, and has been employed extensively in investigations of cannabinoid pharmacology.

Studies by Huffman et al. established that the aminoalkyl portion of the molecule could be replaced by a straight chain alkyl group to provide indole derivatives that also exhibit typical cannabinoid pharmacology [12, 13]. One of the early compounds prepared by this group, JWH-007, 1-pentyl-2-methyl-3-(1-naphthoyl)indole (**5**), has high affinity for the cannabinoid CNS receptor and exhibits typical cannabinoid pharmacology in vivo. JWH-015, the 1-propyl analog of **5** (**6**), exhibits selective affinity for the cannabinoid receptor expressed primarily in the immune system [14]. Lainton et al. found that the benzene ring of the indole is not essential for either receptor affinity or in vivo effects [13, 15]. Cannabimimetic pyrrole derivatives (**7**, R = various straight chain alkyl groups) were reported and although these pyrrole derivatives have relatively modest affinity for the cannabinoid receptors, the *n*-pentyl analog (**7**, R = *n*-C_5H_{11}) is nearly as potent in vivo as THC.

Several aminoalkylindenes structurally related to the cannabimimetic AAIs have been described, some of which have high affinity for both the CNS and peripheral cannabinoid receptors [16, 17]. In these compounds, the indole nitrogen is replaced with a carbon atom to give **8** and similar compounds (Fig. 2).



5 R = *n*-C_5H_{11}
6 R = *n*-C_3H_7

7

8

Fig. 2 Structures of cannabimimetic indoles, pyrroles, and indenes

2 Pharmacology Methods

Before discussing the SAR and receptor interactions of these series of compounds, a brief description of some of the more common methods employed to evaluate the pharmacology of cannabinoids will be presented. A cannabinoid receptor in rat brain was first described by Devane et al. in 1988 [18]. This G-protein-coupled receptor has been cloned and its primary structure has been elucidated [19]. A human cannabinoid CNS receptor has been identified that is essentially identical (97% homology) to that of the rat [20]. It is generally accepted that the overt physiological effects of cannabinoids, including their psychoactive, antinausea, and appetite-stimulating effects, are mediated through this receptor [21, 22]. In 1993, a second human cannabinoid receptor, which is found primarily in the immune system was identified and cloned [23, 24]. The transmembrane portion of this receptor shows 66% identity to the CNS receptor with an overall homology of 44%. The CNS receptor is designated as the CB1 receptor, and that receptor found principally in the immune system is described as the CB2 receptor. CB1 receptor affinity measures the ability of the substrate to displace a potent cannabinoid, usually either tritiated CP-55,940 (**2**) [21], or tritiated WIN-55,212-2 (**4**) [11] from their binding sites in a membrane preparation. Affinity for the CB2 receptor is determined by the ability of a ligand to displace CP-55,940 (**2**) from its binding site in transfected cell lines [14, 23, 25] or a mouse spleen membrane preparation [26]. Alternatively, the in vitro evaluation of cannabinoid activity may employ the inhibition of electrically evoked contractions of the isolated mouse vas deferens [27].

Functional assays are used to evaluate the efficacy of cannabinoid receptor ligands at both the CB1 and CB2 receptors. One of these functional assays measures the agonist-induced attenuation of the ability of forskolin to stimulate the production of cAMP [27]. Another assay determines G-protein-coupled receptor activation using [^{35}S]GTPγS binding [28]. This procedure measures the ability of a cannabinoid receptor ligand to stimulate GTPγS binding.

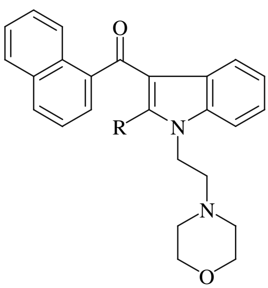
The most widely used procedure for in vivo pharmacological studies is a mouse model in which three or four behavioral procedures are employed [22]. These protocols measure spontaneous activity (SA), antinociception (as tail flick, TF), hypothermia (as decrease in rectal temperature, RT), and catalepsy (as ring immobility, RI). A number of other procedures, particularly drug discrimination studies in various species have been employed to evaluate in vivo pharmacology, however, the mouse model is widely accepted, and this protocol has been used to evaluate the potency of the majority of the compounds described in this chapter that were evaluated in vivo. An extensive review of cannabinoid receptors and the various bioassays used in this field was published in 2002 [29].

3 Cannabimimetic Indoles

3.1 Aminoalkylindoles

Following the discovery that the aminoalkylindole, pravadoline (**3**), inhibited contractions of the electrically stimulated MVD and inhibited prostaglandin synthetase activity, the Sterling group synthesized a number of additional compounds structurally related to pravadoline (Fig. 3) [10]. It was found that four analogs of **3** that lacked prostaglandin synthetase activity still showed antinociceptive activity and inhibited contractions of the electrically stimulated MVD. In all four of these compounds the substituted phenyl moiety present in pravadoline was replaced by a bicyclic aromatic ring. Three of these AAI derivatives have the *p*-methoxybenzoyl group of pravadoline replaced by a 1-naphthoyl substituent and two of these analogs (**9**, **10**) were particularly effective in the MVD inhibition protocol. It was suggested that these data, combined with the observation that AAIs inhibit adenylate cyclase and interact with a G-protein-coupled receptor in the brain, could be indicative of a binding site on the cannabinoid brain receptor.

Subsequently, a series of sterically constrained AAIs (**11**) was prepared, and it was found that the most effective compound in MVD activity was WIN-55,212-2 (**4**) [11]. These analogs were evaluated in an AAI-binding assay, in which the ability of the ligand to displace tritiated **4** from its binding site in a rat brain membrane preparation was determined. There was a positive correlation between these binding data and the MVD assay, and it was concluded that the biological properties, both of the AAIs and traditional cannabinoids were very similar. It was also found that the activity of **4** is restricted to the *R*-enantiomer (depicted); the *S*-isomer was orders of magnitude less potent in both the AAI-binding assay and the MVD protocol. Some preliminary SAR for the AAIs were presented, including the observation that potency was attenuated by substitution at the 2-position of the indole. An AAI with an indole 2-methyl substituent is usually slightly less potent than its unsubstituted congener



9 R = H
10 R = CH_3

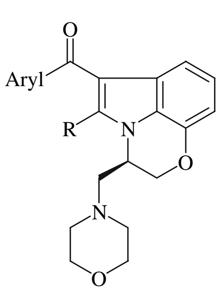


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Fig. 3 Structures of representative aminoalkylindoles

and larger C-2 substituents greatly reduce potency. It was also concluded that a bicyclic 3-substituent, such as 1-naphthoyl, significantly enhanced potency relative to a benzoyl or substituted benzoyl group. It was also found that a two-carbon linker between the indole nitrogen and the nitrogen of the heterocycle containing the amino group enhances potency [11].

Kuster et al. determined the affinities of several AAIs and a number of other classes of biologically active compounds for the WIN-55,212-2 binding site, and it was found that of the more than 60 compounds that were evaluated only traditional cannabinoids competed with [^{3}H] WIN-55,212-2 at the binding site [30]. Receptor affinities as determined by Kuster et al. for selected examples of AAIs and traditional cannabinoids are included in Table 1. These compounds were also found to exhibit typical cannabinoid pharmacology in the standard cannabinoid behavioral protocol [31]. These data are also included in Table 1.

In a subsequent publication, the Sterling group reported the preparation, inhibition of [^{3}H]WIN-55,212-2 binding and inhibition of electrically induced contractions of the isolated mouse vas deferens for 143 aminoalkylindoles and detailed structural requirements for cannabinoid-binding activity were outlined [32]. Earlier studies had concluded that at C-2 of the indole nucleus smaller groups enhance MVD activity and receptor binding; specifically, hydrogen is superior to methyl and an ethyl group greatly attenuates potency [11]. Also, a bicylic aroyl substituent at C-3 of the indole greatly enhances potency. In the 1995 publication the authors described the development of these cannabimimetic indole derivatives and elaborated upon their previously reported SAR to include variations in the nature of the aminoalkyl substituent, the substituents on the naphthoyl group, and substituents on the indole nucleus.

It was found that locating the nitrogen of the amino group farther than two carbon atoms from the indole nitrogen diminished activity, and acyclic amine derivatives were devoid of activity. Of the cyclic amines, morpholine,

Table 1 CB1 receptor affinities and in vivo potencies for selected AAIs and traditional cannabinoids [30, 31]

		ED_{50} (μmole/kg)			
Compound	K_i (nM)	SA	TF	RT	RI
(+)-WIN-55,212-2 (**4**)	2.2 ± 0.3	0.25	0.82	22.96	2.10
(−)-WIN-55,212-2	7.4 ± 0.2	>57.4	>57.4	>57.4	>57.4
Pravadoline (**3**)	2511 ± 177	>20.9	>20.9	>20.9	>20.9
Naphthoylindole (**9**)	ND	0.75	0.68	3.12	7.02
Naphthoylindole (**10**)	16 ± 1	5.02	4.77	18.07	9.79
Δ^9-THC (**1**)	10.2 ± 0.8	2.86	2.54	7.31	7.02
Δ^8-THC	16.5 ± 0.6	2.9[a]	4.8[a]	4.5[a]	4.8[a]
CP-55,940 (**2**)	0.35 ± 0.04	0.11[b]	0.23[b]	0.93[b]	0.92[b]

[a] Martin BR, Compton DR, Semus SF, Lin S, Marciniak G, Grzybowska J, Charalambous A, Makriyannis A. Pharmacological evaluation of iodo and nitro analogs of Δ^8-THC and Δ^9-THC. Pharmacol Biochem Behav. 1993;46:295–301.
[b] Ref. 22

thiomorpholine, and piperidine increased potency. A limited number of 4-substituted-1-naphthoyl derivatives were explored, and there was relatively little effect on the displacement of WIN-55,212-2, although electron-donating substituents (CH_3 and OCH_3) slightly increased potency. Substitution at the 5-position of the indole nucleus decreased activity, and in the limited number of examples cited a 7-substituent improved binding. Also, forming a ring between the 1- and 7-positions of the indole, as in WIN-55,212-2 (**4**), greatly increased binding.

The 6-bromo derivative of pravadoline (**12**) showed some antagonist behavior in vitro, but not in vivo. Subsequently, the Makriyannis group prepared 6-iodopravadoline (**13**, AM630), which shows antagonist, or inverse agonist, properties in vitro [33–35]. Further investigation demonstrated that AM630 has complex pharmacological properties in that it is a weak partial agonist at the CB1 receptor with $K_i = 5152 \pm 567$ nM, but an inverse agonist at the CB2 receptor with $K_i = 31.2 \pm 12.4$ nM [36]. The inverse agonist/antagonist properties of AM630 have found considerably utility in the investigation of CB2 receptor-mediated pharmacological effects, in particular in the study of pain in the periphery.

The Sterling group suggested a pharmacophore for the AAIs that included three key structural elements: an amino nitrogen atom in the side chain appended to the indole nitrogen, an aroyl substituent at C-3 of the indole combined with an indole nucleus [32]. All of the AAI agonists described by these authors fit this pharmacophore, however, some compounds that fit the pharmacophore are not active. In an alternative approach to modeling these compounds the structure of WIN-55,212-2 (**4**) was overlapped with that of Δ^9-THC (**1**). The alignment that was suggested assumed that the phenolic hydroxyl of THC and the amino nitrogen of **4** have a hydrogen bonding interaction with the same point on the receptor. The lipophilic side chain of the traditional cannabinoid and the 3-aroyl substituent of the indole were considered to overlap in this alignment (Fig. 4) [32].

In subsequent work the AAI structure was modified and the two-carbon unit linking the indole nitrogen with the heterocyclic amino group was replaced with

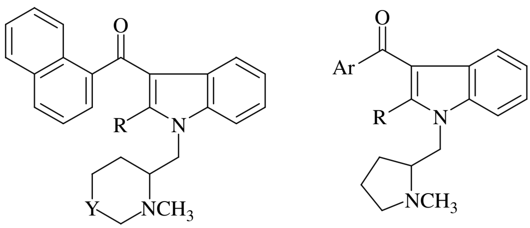


Fig. 4 Structures of representative C-attached aminoalkylindoles

14 R = CH_3, Y = CH_2
15 R = H, Y = CH_2
16 R = H, Y = O

17 R = CH_3, Ar = 1-naphthyl
18 R = H, Ar = *p*-OCH_3-phenyl

a one-carbon unit, which is attached to the carbon atom adjacent to the amino group [37]. This structural modification maintains the two-carbon linker between the indole nitrogen and the amino group that is necessary for optimum cannabinoid activity. In common with other AAIs the 3-(1-naphthoyl) analogs have higher affinity for the CB1 receptor than those with a substituted benzoyl group at the indole 3-position. Six naphthoyl indole analogs were reported, five of which (**14** to **17**) have IC_{50} values below 10 nM in the [^{3}H]WIN-55,212-2 binding assay. These five are also quite potent in the mouse vas deferens procedure. Unexpectedly, one compound in this series in which the aroyl substituent is *p*-methoxybenzoyl (**18**) also has high affinity in the [^{3}H]WIN-55,212-2 binding assay and is potent in the mouse vas deferens procedure. The highest affinity ligand in this series is a 2-(1-methylpiperidinyl) analog (**15**). The [^{3}H]WIN-55,212-2 binding for the racemate of this compound was reported as $IC_{50} = 1.22 \pm 0.02$ nM. The compound was resolved by chiral HPLC and one enantiomer has $K_i = 0.27$ nM, while the enantiomer has $K_i = 217$ nM. The absolute stereochemistry of these enantiomers was not reported.

Indoles **14** to **18** were included in a comparative molecular field analysis (CoMFA) of a number of AAIs that was carried out to develop a 3D-quantitative SAR (3D-QSAR) model for this class of cannabinoids [38]. This CoMFA study correlates structural variations in these ligands with their receptor affinities. CoMFA constructs a 3D-QSAR model by correlating steric and electrostatic fields for various structures with the observed binding affinities for each ligand. Two models were designed, one of which employed receptor affinities for a series of 14 AAIs, determined by measuring the displacement of [3H]CP-55,940 (**2**) from a rat brain membrane preparation (CoMFA model 1). The other model used the displacement of [3H]WIN-55,212-2 (**4**) from the same rat brain preparation by 64 AAIs (CoMFA model 2). The CoMFA-predicted pK_i values agreed quite well with those determined experimentally and the authors suggested that although classical cannabinoids and AAIs are structurally very different, they occupy substantially similar areas of the CB1 receptor. The CB1 binding data presented by Shim et al. were reported as pK_i values ($-\log K_i$) and the CP-55,940 and WIN-55,212-2 binding data for indoles **9**, **10** and **14** to **18** are summarized in Table 2. For comparison with receptor affinities reported elsewhere in this chapter the pK_i values have been converted to K_i.

From the data presented in Table 2 it is apparent that CB1 receptor binding data obtained by the displacement of [^{3}H]CP-55940 and [^{3}H]WIN-55,212-2 are not identical [38]. The authors concluded that, "... it is clearly possible and highly likely based on structural comparisons that each class of compounds exhibits unique interactions within the receptor." This conclusion is consistent with experimental data reported by Song and Bonner in which a lysine is required for the interaction of traditional cannabinoids, but not AAIs with the CB1 receptor [40].

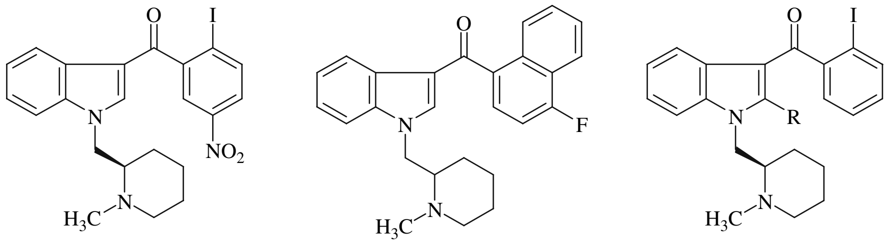
Based upon the CB1 receptor, affinities of the C-attached AAIs described in Table 2 substitution of the 2-(4-morpholino)ethyl group in **9** and **10** with a [2-(1-methylpiperidinyl)] substituent leads to a considerable increase in CB1

Table 2 Binding parameters for C-attached AAIs [39] and selected ligands used in a CoMFA study of the AAIs [38]

Compound	IC_{50} (displacement of **4**)	K_i (nM)[a] (displacementof **2**)	K_i (nM)[a] (displacement of **4**)
Pravadoline (**3**)	3155 ± 54[b]	ND	2512
WIN-55,212-2 (**4**)	ND	2.19	1.11
9	7.8 ± 0.3[b]	47.9	6.02
10	19 ± 2[b]	28.2	15.1
14	5.4 ± 0.9[c]	ND	4.26
15	1.22 ± 0.02[c]	0.76	1.05
16	3.0 ± 0.16[c]	10.7	2.40
17	6.5 ± 0.6[c]	ND	5.13
18	9.7 ± 1.7[c]	ND	7.58

[a] Ref. 38, K_i values were calculated from the reported pK_i data
[b] Ref. 32
[c] Ref. 37

receptor affinity [37]. This aminoalkyl unit was exploited by the Makriyannis group in the design of the highly selective CB2 receptor ligand AM1241, (2-iodo-5-nitrophenyl)-[1-(1-methylpiperidin-2-ylmethyl)-1*H*-indol-3-yl]methanone (**19**, *R*-enantiomer depicted) with $K_i = 3.4 \pm 0.5$ nM at the CB2 receptor and $K_i = 280 \pm 41$ nM at the CB1 receptor [40, 41]. These binding data were obtained using a mouse spleen preparation for determining the CB2 affinity and a rat brain preparation for the CB1 receptor affinity and are presumably for the racemate [40]. In the course of the original preparation of AM1241 the compound was resolved by chiral HPLC and the absolute stereochemistry was determined by X-ray crystallography. It was found that the *R*-(+)-enantiomer, AM1241-1 (**19**), has higher affinity for both the CB1 and CB2 receptors than the *S*-(−)-enantiomer (AM1241-2, not depicted) [42]. Presumably, the more potent enantiomer of the naphthoyl analog of AM1241, indole **15**, also has the *R* configuration (Fig. 5) [37].



19 **20** **21** R = CH_3 **22** R = H

Fig. 5 Structures of AM1241 and aminoalkylindoles used in radiography

AM1241 has been employed in several studies and has been found to produce antinociception to thermal stimuli, an effect that is blocked by the CB2 receptor antagonist AM630 (**13**) [40, 43]. Also, it was found that the antihyperalgesic and antialloldynic effects of AM1241 were blocked by the CB2 antagonist SR144528, but not by the CB1 antagonist SR14176A [42]. Similar effects were noted in capsaicin-induced hyperalgesia and alodynia [44]. These effects are attributed to the peripheral release of endogenous opiods by CB2 receptor activation [45]. This selective agonist for the CB2 receptor was also employed in a study of the identification and functional characterization of CB2 receptors in the brain [46]. Although neither AM1241 nor another highly selective CB2 agonist, JWH-133, could inhibit emesis in the ferret, AM1241 in combination with the endogenous CB1 cannabinoid receptor ligand anandamide did inhibit emesis at a dose of anandamide that was ineffective when used alone.

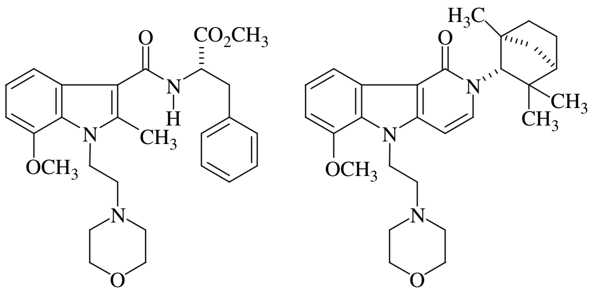
In an approach to the preparation of CB1 receptor ligands suitable for positron emission tomagraphy (PET) Willis et al. described the synthesis and SAR for a series of analogs of **15** in which the 1-naphthyl moiety contained substituents at the 2-, 4- or 6-positions [47]. The 4-substituents included methyl, fluoro, bromo, cyano, nitro, 4-(2-hydroxyethyl) and hydroxyl. 2-Methyl-1-naphthyl, 6-hydroxy-1-naphthyl and 2-iodophenyl analogs were also prepared. The compound with an unsubstituted naphthyl substituent (**15**) had been described previously [39]. All of these compounds were prepared as racemates and the 4-nitro-1-naphthyl and 2-iodophenyl compounds had been described by Deng [41]. The goal of these authors was the development of a relatively nonlipophilic CB1 receptor ligand that could be isotopically labeled with an appropriate radioactive atom suitable for PET imaging. One of the compounds prepared by Willis et al., the 4-fluoro-1-naphthyl analog (**20**), has high affinity for the CB1 receptor ($K_i = 0.7 \pm 0.1$ nM) and was subsequently labeled with ^{18}F, which has a half-life of 110 min Racemic **20** showed moderate uptake into the whole mouse brain, with a pattern of distribution of the ligand that was consistent with the known distribution of CB1 receptors in the brain. The enantiomers of indole **20** were separated by chiral HPLC and it was found that the ^{18}F labeled (+)– enantiomer was the stereoisomer that showed significant specific binding in the mouse brain.

An alternative approach to radiolabeled aminoalkylindole cannabinoid receptor ligands was described by Deng et al. [48]. In this study a series of analogs of indole **14**, **15,** and **19** were synthesized, all of which contained an iodo substituent. The goal was the development of a high-affinity ligand or ligands that would contain one of the radioisotopes of iodine and which would be suitable for in vitro autoradiography or in vivo single photon emission computed tomography (SPECT). The highest affinity ligands are the iodobenzoyl analogs **21** and **22,** both of which were initially prepared as racemates and then as the individual enantiomers. As the racemate, the 2-methyl homolog (**21**) has $K_i = 34$ nM at both the CB1 and CB2 receptor. The indole that is unsubstituted at C-2 (**22**) has higher affinity as the racemate for both receptors with $K_i = 2.8$ nM at the CB1 receptor and $K_i = 2.9$ nM at the CB2 receptor. Resolution of

both compounds indicated that the (+)-enantiomer was far more potent than its optical antipode. For (+)-**21**, $K_i = 6.7$ nM at the CB1 receptor and $K_i = 10$ nM at the CB2 receptor, while for (−)-**21** $K_i = 1200$ nM at the CB1 receptor and $K_i = 83$ nM at the CB2 receptor. For (+)-**22**, $K_i = 1.80$ nM at the CB1 receptor and $K_i = 2.2$ nM at the CB2 receptor, while for (−)-**22** $K_i = 560$ nM at the CB1 receptor and $K_i = 580$ nM at the CB2 receptor. The absolute configurations of the individual enantiomers of **22** were determined by their synthesis from the respective enantiomers of pipecolinic acid. It was found that the very potent (+)-enatiomer of **22** has the *R*-configuration as depicted in structure **22**. For AM1241 (**19**) the *R*-enantiomer also has significantly higher affinity for both receptors than the *S*-enantiomer [41].

Both enantiomers of aminoalkylindole **22** containing ^{131}I (half-life of 8 d) were synthesized and were evaluated using autoradiography in mouse brain. [^{131}I](*R*)-**22** gave images consistent with binding to CB1 receptors in the brain. Binding of [^{131}I](*S*)-**22** was much less than that of the labeled racemate [48]. Also, binding of [^{131}I](*R*)-**22** was not observed in brain sections prepared from a CB1 receptor knockout mouse, indicating that the in vitro binding was to cannabinoid CB1 receptors. These authors note that the CB1 and CB2 receptor affinities of (*R*)-**22** are very similar, but that this lack of selectivity should present no problems in using this compound in brain imaging due to the lack of CB2 receptors in the brain.

Two novel series of aminoalkylindoles were developed by a group at Bristol-Myers Squibb (Fig. 6). One series consisted of amides derived from 7-methoxyindole 3-carboxylic acid and several of these compounds have good affinity for the CB2 receptor [49]. The only compound in this series for which CB1 binding data were reported was a highly selective phenylalanine-derived amide (**23**), which has excellent affinity for the CB2 receptor ($K_i = 8$ nM) and little affinity for the CB1 receptor ($K_i = 4000$ nM). Several of the compounds described in this report were *S*-fenchyl amides, which have from modest to good affinity for the CB2 receptor. This observation was used to design a second series of cannabimimetic indoles, which are pyridones derived from compounds similar to **23** [50]. One of these indolopyridones (**24**) has very high affinity for the CB2 receptor ($K_i = 1.0 \pm 0.2$ nM), but also has high affinity for the CB1



23 24

Fig. 6 Bristol-Meyers Squibb cannabimimetic indoles

receptor ($K_i = 16 \pm 4$ nM). Indolopyridone **24** is orally effective in a mouse model of inflammation.

3.2 Alkylindoles

The aminoalkylindole pharmacophore suggested by Eissenstat et al. requires three structural elements: a heterocyclic amino nitrogen attached to the indole nitrogen by a two-carbon linker, an aroyl group at the indole C-3 position, and an indole nucleus [32]. These authors also proposed that in binding to the CB1 receptor, the phenolic hydroxyl of THC (**1**) and the amino nitrogen of WIN-55,212-2 (**4**) have hydrogen-bonding interactions at the same point on the receptor. The 3-aroyl group of the indole and the lipophilic side chain of THC would overlap in this alignment. An alternative alignment was proposed by Shim et al. in which the indole carbonyl of **4** and the phenolic hydroxyl group of traditional cannabinoids are involved in a hydrogen-bonding interaction with Lys 192 of the CB1 receptor [51]. In this hypothetical model, the naphthalene ring corresponds to the lipophilic side chain of THC and the indole nucleus is in juxtaposition with the cyclohexene ring of THC. This model was derived from the CoMFA study mentioned above [38].

Huffman et al. suggested a very different alignment of WIN-55,212-2 (**4**) and Δ^9-THC (**1**) based upon the observation that the ketonic carbonyl of **4**, which has the properties of a vinylogous amide, corresponds to the phenolic hydroxyl of traditional cannabinoids, and would be an effective hydrogen bond acceptor [12]. This hypothesis was used in a molecular modeling study in which the phenolic hydroxyl of THC (**1**) was overlaid upon the ketonic carbonyl of WIN-55,212-2 (**4**) and the naphthalene ring of **4** was aligned with the carbocyclic A-ring of THC. Specifically, C-9 and C-10 of THC and naphthalene carbons 7 and 8 of WIN-55,212-2 were overlapped (for numbering, see structures **1** and **4**). In this alignment the indole nitrogen corresponds to the benzylic carbon (C-1′) of THC and the aminoalkyl group of **4** overlaps the alkyl side chain of THC. This alignment led to the prediction that an aminoalkyl group was not essential for cannabinoid activity, and that it could be replaced with an alkyl group. A flaw in both this and the alignment suggested by Shim [51] lies in the observation by Song and Bonner that in a mutant receptor in which Lys 192 was replaced by an alanine and that the binding of traditional cannabinoids was greatly attenuated, while that of WIN-55,212-2 was effectively unchanged [39].

Using their proposed alignment, Huffman et al. synthesized a series of ten, 1-alkyl-2-methyl-3-(1-naphthoyl)indoles (**25**) and evaluated their CB1 receptor affinities and in vivo pharmacology [12]. Those 1-alkyl-2-methyl-3-(1-naphthoyl)indoles with nitrogen substituents of four to six carbon atoms (**25**, R = *n*-butyl through *n*-hexyl, 2-heptyl and 2-cyclohexylethyl) have from moderate to high CB1 receptor affinities with $K_i = 9.5$–81 nM. The in vivo pharmacology for these compounds was

reported as the average of the data from the mouse model and the behavioral data were consistent with the receptor affinities of these cannabimimetic indoles. 1-Pentyl-2-methyl-3-(1-naphthoyl)indole (JWH-007, **25**, R = *n*-pentyl) with $K_1 = 9.5 \pm 4.5$ nM has receptor affinity approximately five times that of THC, and is nearly twice as potent in vivo.

To investigate the SAR for these cannabimimetic indoles a series of related 1-alkyl-3-(1-naphthoyl)indoles (**26**) was prepared and their pharmacology was evaluated. Wiley et al. combined these data with a description of the detailed pharmacology for the 1-alkyl-2-methyl-3-(1-naphthoyl)indoles (**25**) and presented a discussion of the SAR for 22 indole derivatives [13]. For both the 2-methylindole analogs (**25**) and those compounds lacking the 2-methyl group (**26**) the 1-methyl and 1-ethyl indoles have no affinity for the receptor, while the 1-butyl, 1-pentyl, and 1-hexyl derivatives have from moderate to high affinity for the CB1 receptor and exhibit typical cannabinoid pharmacology in the mouse with a full spectrum of dose-related effects. The pharmacology of representative examples of indoles **25** and **26**, plus WIN-55,212-2 (**4**) and Δ^9-THC (**1**) are summarized in Table 3.

Although cannabimimetic indoles **25** and **26** are not as effective in the RT measure of hypothermia as traditional cannabinoids with comparable receptor affinity they show a typical spectrum of cannabinoid pharmacology [13]. Several of these compounds were evaluated in drug discrimination experiments in rats and dose-dependent substitution for Δ^9-THC was observed. In the 2-methyl series (**25**) CB1 receptor affinity increased as the nitrogen substituent was varied from 1-methyl to 1-pentyl and then decreased from 1-hexyl to 1-heptyl. In all cases in vivo pharmacology was consistent with receptor affinity. The *N*-heptyl derivative of **25** (JWH-009, $R = C_7H_{15}$) has no affinity for the receptor ($K_i \geq 10{,}000$), and is completely inactive in vivo. This 1-heptyl compound was also evaluated in a drug discrimination procedure in rhesus monkeys and did not substitute for THC, although the corresponding butyl

Table 3 CB1, CB2 receptor affinities and in vivo potencies of selected cannabimimetic indoles [13, 56]

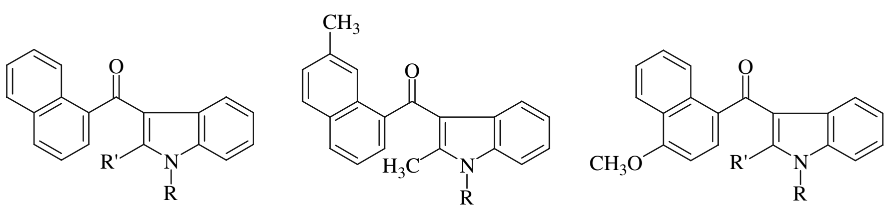
	K_i (nM)			ED_{50} (μmole/kg)		
Compound	CB1	CB2	SA	MPE[a]	RT	RI
25, $R = C_3H_7$, JWH-015	164 ± 22	13.8 ± 4.6	18.7	84.7	99.1	87.2
25, $R = C_4H_9$, JWH-016	22 ± 1.5	4.3 ± 1.6	2.6	0.23	4.1	12.9
25, $R = C_5H_{11}$, JWH-007	9.5 ± 4.5	2.9 ± 3.0	0.70	0.25	4.3	1.9
25, $R = C_6H_{13}$, JWH-004	48 ± 13	4.0 ± 1.5	<2.7	9.5	17.1	16.0
25, $R = C_7H_{15}$, JWH-009	>10,000	141 ± 14	117	>261	>261	>261
26, $R = C_3H_7$, JWH-072	1050 ± 5.5	170 ± 54	ND	ND	ND	ND
26, $R = C_4H_9$, JWH-073	8.9 ± 1.8	27 ± 12	0.34	1.3	3.3	NT
26, $R = C_5H_{11}$, JWH-018	9 ± 5	2.9 ± 3.0	0.44	~0.09	1.7	3.9
26, $R = C_6H_{13}$, JWH-019	9.8 ± 2	5.6 ± 2.0	0.96	0.73	1.5	ND
26, $R = C_7H_{15}$, JWH-020	128 ± 17	205 ± 20	56.9	17.6	>81.3	ND

[a] Percent maximum possible antinociceptive effect in the TF assay

compound, JWH-016 (**25**, R = C_4H_9), produced full dose-dependent substitution for THC [52]. The 1-pentyl analog JWH-007 (**25**, R = C_5H_{11}) also produced a dose-related inhibition of electrically induced contractions in the isolated mouse vas deferens [53]. This behavior was inhibited by the cannabinoid receptor antagonist SR-141716A, which provided additional evidence that indoles **25** and **26** are cannabinoid receptor agonists.

Although in general the 2-methylindoles have lower affinity for the CB1 receptor than those indoles unsubstituted at C-2 the CB1 receptor affinity for the 1-propyl compound, JWH-072 (**26**, R = C_3H_7), was considerably diminished relative to the 2-methyl analog, JWH-015 (**25**, R = C_3H_7). For **26**, R = C_3H_7, $K_i = 1050 \pm 55$ nM) and for the corresponding 1-propyl-2-methyl indole, JWH-015 (**25**, R = C_3H_7) $K_i = 164 \pm 22$ nM [13]. In contrast to 1-heptyl indole JWH-009 (**25**, R = C_7H_{15}) the heptyl analog of **26**, JWH-020 (R = C_7H_{15}) has modest affinity for the CB1 receptor ($K_i = 128 \pm 17$), shows typical cannabinoid pharmacology in vivo [13], and produces a dose-related inhibition of electrically invoked contractions in the isolated mouse vas deferens [54]. The *N*-butyl (JWH-073, **26**, R = C_4H_9), *N*-pentyl (JWH-018, **26** R = C_5H_{11}) and *N*-hexyl (JWH-019, **26**, R = C_6H_{13}) indoles lacking a methyl group at the indole 2-position (**26**) have essentially the same affinity for the CB1 receptor (K_i = ~9 nM), and all three analogs have similar potency in the in vivo the mouse protocol [13].

In an early study directed toward an understanding of SAR for the CB2 receptor, Showalter et al. determined the CB1 and CB2 affinities of a number of diverse cannabinoid receptor agonists (Fig 7) [14]. Although traditional cannabinoids, such as THC (**1**), have approximately equal affinity for both receptors, three cannabimimetic indoles, WIN-55,212-2 (**4**), JWH-015, 1-propyl-2-methyl-3-(1-naphthoyl)indole (**6** or **25**, R = C_3H_7), and JWH-018, 1-pentyl-3-(1-naphthoyl)indole (**26**, R = C_5H_{11}) have greater affinity for the CB2 receptor than for the CB1 receptor. WIN-55,212-2 (**4**) and 1-pentyl-3-(1-naphthoyl)indole (**26**, R = C_5H_{11}) both have high affinity for the CB1 receptor; however, JWH-015 (**6** or **25**, R = C_3H_7) does not ($K_i = 164 \pm 22$ nM), although it does have high affinity for the CB2 receptor ($K_i = 13.8 \pm 4.6$ nM). In an investigation of ligands



25 R = alkyl, R' = CH_3
26 R = alkyl, R' = H

27 R = alkyl

28 R = alkyl, R' = H
29 R = alkyl, R' = CH_3

Fig. 7 Structures of cannabimimetic *N*-alkylindoles

that are selective for the CB2 receptor, it was found that JWH-015 inhibits the electrically induced contractions of the guinea pig myenteric plexus preparation and mouse vas deferens [54]. Although the inhibition in the myenteric plexus preparation was attenuated by the CB1 selective antagonist, SR-141716A, the inhibition in the mouse vas deferens was not, at least at low concentrations of the antagonist. It was suggested that in the myenteric plexus preparation, inhibition of the twitch response is due to interactions with the CB1 receptor, while in the vas deferens both CB1 and CB2 receptors may mediate the electrically evoked contractions [55]. In subsequent work, the [^{35}S]GTPγS assay indicated that this compound (JWH-015) was potent with $EC_{50} = 17.7 \pm 1.0$ nM, but was a partial agonist with $E_{max} = 65.7 \pm 6.4\%$ relative to CP55,940 [55].

To obtain additional CB2 selective ligands and to investigate the SAR for cannabimimetic indoles at the CB1 and CB2 receptors, the Clemson group synthesized a number of additional 3-(1-naphthoyl)indoles and evaluated their pharmacology [55, 56]. The initial series of compounds included 1-alkyl-2-methyl-3-(7-methyl-1-naphthoyl)indoles (**27**), 1-alkyl-3-(4-methoxy-1-naphthoyl)indoles (**28**), and 1-alkyl-2-methyl-3-(4-methoxy-1-naphthoyl)indoles (**29**). In the 1-alkyl-3-(4-methoxy-1-naphthoyl)indole series the 1-methyl (**28**, R = CH_3) and 1-ethyl (**28**, R = C_2H_5) compounds have virtually no affinity for the CB1 receptor with $K_i \geq$ 10,000 nM and 817 ± 60 nM, respectively [56]. The CB1 receptor affinity in all three series (**27** to **29**) increases from R = C_3H_7 to R = R_5H_{11}, then decreases to R = C_7H_{15}. Receptor affinity and limited in vivo data for several of these compounds are summarized in Table 4.

For the 7-methyl analogs (**27**) the presence of the additional substituent has little effect upon the affinity for the CB1 receptor, however the *n*-heptyl analog, JWH-050 (**27**, R = C_7H_{15}), has slight affinity with $K_i = 342 \pm 61$ nM, while the analog lacking the naphthyl 7-methyl group, JWH-009 (**25**, R = C_7H_{15}), has $K_i \geq$10,000 nM at the CB1 receptor [56]. Analogous to the 1-propyl analog of **25**, the *N*-propyl derivative of **27** (JWH-046, R = C_3H_7) is also a selective ligand

Table 4 CB1, CB2 receptor affinities and in vivo potency for selected naphthyl-substituted cannabimimetic indoles [56]

	K_i (nM)		ED_{50} (μmole/kg)		
Compound	CB1	CB2	SA	TF	RT
27, R = C_3H_7, JWH-046	343 ± 38	16 ± 5	No max	No max	No max
27, R = C_4H_9, JWH-047	59 ± 3	0.9	No	dose responsive	
27, R = C_5H_{11}, JWH-048	11 ± 1	0.5 ± 0.1	<2.7	<2.7	<2.7
27, R = C_6H_{13}, JWH-049	55 ± 17	6	<7.8	Nomax	No max
27, R = C_7H_{15}, JWH-050	342 ± 61	424	No max	No max	No max
28, R = C_3H_7, JWH-079	63 ± 3	32 ± 6	5.5	10.0	12.3
28, R = C_4H_9, JWH-080	7.6 ± 1.0	ND	7.8	4.3	2.4
28, R = C_5H_{11}, JWH-081	1.2 ± 0.1	ND	0.2	0.2	0.2
28, R = C_6H_{13}, JWH-082	5.3 ± 0.8	6.4 ± 0.9	4.5	1.6	3.0
29, R = C_3H_7, JWH-094	476 ± 67	97 ± 3	0.96	0.73	1.5
29, R = C_4H_9, JWH-096	34 ± 3	ND	ND	ND	ND
29, R = C_5H_{11}, JWH-098	4.5 ± 0.1	ND	ND	ND	ND

for the CB2 receptor with little affinity for the CB1 receptor. To determine the effect of a 2-methyl group on CB2 selectivity, the affinity at both receptors for JWH-072 (**26**, $R = C_3H_7$) and an analog of **27** (JWH-072, $R = C_3H_7$) but unsubstituted at C-2 of the indole 2-position were determined. The 1-propyl analog of **26** (JWH-072, $R = C_3H_7$) has very modest affinity for the CB2 receptor with $K_i = 170 \pm 54$ nM. The analog of **27** (JWH-076, $R = C_3H_7$) but unsubstituted at C-2 of the indole also has modest affinity for the CB2 receptor ($K_i = 106 \pm 46$ nM) and only slightly less affinity for the CB1 receptor with $K_i = 214 \pm 11$ nM [56].

In the 4-methoxy-1-naphthoyl series those compounds unsubstituted at C-2 (**28**, $R = C_3H_7$ to C_7H_{16}) in general have somewhat higher affinity for the CB1 receptor than the corresponding naphthoyl indoles lacking substitution on the naphthalene ring (**26**, $R = C_3H_7$ to C_7H_{16}) [57]. The compounds in the 2-methyl series (**29**, $R = C_3H_7$ to C_7H_{16}) have from slightly less to considerably less affinity for the CB1 receptor than the C-2 unsubstituted indoles (**28**, $R = C_3H_7$ to C_7H_{16}). Indoles **29**, $R = C_3H_7$ to C_7H_{16}, all show selectivity for the CB2 receptor relative to the CB1 receptors, however, none of them have the combination of high CB2 receptor affinity combined with little affinity for the CB1 receptor. For the compounds unsubstituted at the indole C-2 position, neither the 4-methoxy-1-naphthoyl series (**28**, $R = C_3H_7$ to C_7H_{16}) nor the 1-naphthoyl compounds (**26**, $R = C_3H_7$ to C_7H_{16}) exhibit significant selectivity for either the CB1 or CB2 receptor. JWH-081, the *N*-pentyl analog of **28**, $R = C_5H_{11}$, has very high affinity for the CB1 receptor with $K_i = 1.2 \pm 0.1$ nM and is very potent in vivo (Table 4). The trend in CB2 receptor affinities for indoles **29**, $R = C_3H_7$ to C_7H_{15}, follows the same pattern as the CB1 affinities, that is, they increase as the length of the *N*-alkyl chain is increased from propyl to pentyl and then decreases with hexyl and heptyl [56].

In a subsequent study, Huffman et al. reported the synthesis, CB1 and CB2 receptor affinities for 47 1-propyl and 1-pentyl-3-(1-naphthoyl)indoles with a variety of substituents appended to the naphthalene ring [55]. The effects of these substituents upon receptor affinities were described and SAR at both receptors were proposed. This study was restricted to the 1-propyl and 1-pentylindoles since it had been noted previously that the two most highly CB2-selective cannabimimetic indoles (**6** and **27**, $R = C_3H_7$) both have an *N*-propyl substituent [14, 56]. The 1-pentyl substituent was chosen because it been found that cannabimimetic indoles with a 1-pentyl group almost invariably have greater CB1-receptor affinity than other *N*-alkyl-substituted indoles [12, 13, 56].

These cannabimimetic indoles included 3-(4- and 7-alkyl-1-naphthoyl)indoles, the 2-, 6-, and 7-methoxy-1-naphthoyl analogs and compounds with a 4-ethoxy-1-naphthoyl substituent [55]. In all cases both the unsubstituted and 2-methyl indole analogs were investigated. The 3-(4-alkyl-1-naphthoyl)indoles (**30**) have straight chain alkyl groups ranging from methyl to butyl. The 7-alkyl analogs (**31**) were analogous to indole **27**, but contained a 7-ethyl group in place

of the 7-methyl substituent present in **27**. The CB1 and CB2 receptor affinities of these compounds are summarized in Table 5.

With the exception of the 1-propyl-4-methyl compounds (**30**, $R = C_3H_7$, $R' = H$ or CH_3, $R'' = CH_3$) the 4-methyl- (**30**, $R = C_5H_{11}$, $R' = H$ or CH_3, $R'' = CH_3$), 4-ethyl- (**30**, C_3H_7 or $R = C_5H_{11}$, $R' = H$ or CH_3, $R'' = C_2H_5$), and 4-propyl -1-naphthoylindoles (**30**, C_3H_7 or $R = C_5H_{11}$, $R' = H$ or CH_3, $R'' = C_3H_7$) (Table 5) all have uniformly high affinities for both receptors. Following the usual trend for CB1 receptor affinities, the 1-propyl compounds have lower affinities than the 1-pentyl analogs, and the compounds with an indole 2-methyl group have somewhat lower affinities than the unsubstituted analogs. Several of these compounds have very high CB1 receptor affinities, in particular, 1-pentyl-3-(4-ethyl-1-naphthoyl)indole, JWH-210 (**30**, $R = C_5H_{11}$,

Table 5 CB1 and CB2 receptor affinities of 3-(alkyl-1-naphthoyl) and 3-(4-halo-1-naphthoyl)indoles [55, 58]

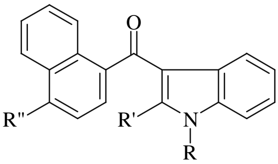
	K_i (nM)	
Compound	CB1	CB2
30, $R = C_3H_7$, $R' = H$, $R'' = CH_3$, JWH-120	1054 ± 31	6.1 ± 0.7
30, $R = C_3H_7$, R', $R'' = CH_3$, JWH-148	123 ± 8	14 ± 1.0
30, $R = C_3H_7$, $R' = H$, $R'' = C_2H_5$, JWH-212	33 ± 0.9	10 ± 1.2
30, $R = C_3H_7$, $R' = CH_3$, $R'' = C_2H_5$, JWH-211	70 ± 0.8	12 ± 0.8
30, $R = C_5H_{11}$, $R' = H$, $R'' = C_2H_5$, JWH-210	0.46 ± 0.03	0.69 ± 0.01
30, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_2H_5$, JWH-213	1.5 ± 0.2	0.42 ± 0.05
30, $R = C_3H_7$, $R' = H$, $R'' = C_3H_7$, JWH-180	26 ± 2	9.6 ± 2.0
30, $R = C_3H_7$, $R' = CH_3$, $R'' = C_3H_7$, JWH-189	52 ± 2	12 ± 0.8
30, $R = C_5H_{11}$, $R' = H$, $R'' = C_3H_7$, JWH-182	0.65 ± 0.03	1.1 ± 0.1
30, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_3H_7$, JWH-181	1.3 ± 0.1	0.62 ± 0.04
30, $R = C_3H_7$, $R' = H$, $R'' = C_4H_9$, JWH-239	342 ± 20	52 ± 6
30, $R = C_3H_7$, $R' = CH_3$, $R'' = C_4H_9$, JWH-241	147 ± 20	49 ± 7
30, $R = C_5H_{11}$, $R' = H$, $R'' = C_4H_9$, JWH-240	14 ± 1	7.2 ± 1.3
30, $R = C_5H_{11}$, $R' = H$, $R'' = C_4H_9$, JWH-242	42 ± 9	6.5 ± 0.3
31, $R = C_3H_7$, $R' = H$, JWH-235	338 ± 34	123 ± 34
31, $R = C_3H_7$, $R' = CH_3$, JWH-236	1351 ± 204	240 ± 63
31, $R = C_5H_{11}$, $R' = H$, JWH-234	8.4 ± 1.8	3.8 ± 0.6
31, $R = C_5H_{11}$, $R' = CH_3$, JWH-262	28 ± 3	5.6 ± 0.7
30, $R = C_3H_7$, $R' = H$, $R'' = F$, JWH-414	240 ± 7	33 ± 2
30, $R = C_3H_7$, $R' = CH_3$, $R'' = F$, JWH-415	530 ± 37	38 ± 1
30, $R = C_5H_{11}$, $R' = H$, $R'' = F$, JWH-412	7.2 ± 0.5	3.2 ± 0.5
30, $R = C_5H_{11}$, $R' = CH_3$, $R'' = F$, JWH-413	14 ± 0.7	2.2 ± 0.2
30, $R = C_3H_7$, $R' = H$, $R'' = Cl$, JWH-400	93 ± 8	44 ± 4
30, $R = C_3H_7$, $R' = CH_3$, $R'' = Cl$, JWH-399	187 ± 16	22 ± 1
30, $R = C_5H_{11}$, $R' = H$, $R'' = Cl$, JWH-398	2.3 ± 0.1	2.8 ± 0.2
30, $R = C_5H_{11}$, $R' = CH_3$, $R'' = Cl$, JWH-397	8.9 ± 0.3	2.3 ± 0.1
30, $R = C_3H_7$, $R' = H$, $R'' = Br$, JWH-386	161 ± 16	27 ± 2
30, $R = C_3H_7$, $R' = CH_3$, $R'' = Br$, JWH-395	372 ± 43	30 ± 2
30, $R = C_5H_{11}$, $R' = H$, $R'' = Br$, JWH-387	1.2 ± 0.1	1.1 ± 0.1
30, $R = C_5H_{11}$, $R' = CH_3$, $R'' = Br$, JWH-394	2.8 ± 0.2	14 ± 0.2

$R' = H$, $R'' = C_2H_5$) with $K_i = 0.46 \pm 0.03$ nM and the 4-propyl analog, JWH-182 (**30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_3H_7$) with $K_i = 0.65 \pm 0.03$ nM. 1-Pentyl-3-(4-methyl-1-naphthoyl)indole, JWH-122 (**30**, $R = C_5H_{11}$, $R' = H$, $R'' = CH_3$), also has subnanomolar affinity for the CB1 receptor ($K_i = 0.69 \pm 0.5$ nM) [55]. Also, the CB2 receptor affinities for the 4-propyl and 4-ethyl compounds fall within a narrow range, from $K_i = 0.62 \pm 0.04$ to $K_i = 12 \pm 0.8$ [56]. Although neither of the 1-propyl-4-methyl compounds (**30**, $R = C_3H_7$, $R' = H$ or CH_3, $R'' = CH_3$) have high affinity for the CB1 receptor, both have good affinity for the CB2 receptor. One of these compounds, 2-methyl-1-propyl-3-(4-methyl-1-naphthoyl)indole, JWH-120 (**30**, $R = C_3H_7$, $R' = CH_3$, $R'' = CH_3$), is a highly selective ligand for the CB2 receptor with $K_i = 6.1 \pm 0.7$ nM at the CB2 receptor and $K_i = 1054 \pm 31$ nM at the CB1 receptor [55].

The 4-butyl-1-naphthoylindoles (Table 5) have considerably lower affinities for the CB1 receptor than the 4-alkyl-1-naphthoylindoles with smaller C-4 substituents [55]. Predictably, the CB1 receptor affinities of the 1-pentyl-3-(4-butyl-1-naphthoyl)indoles are significantly higher than those of the 1-propyl compounds. 1-Pentyl-3-(4-butyl-1-naphthoyl)indole, JWH-240 (**30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_4H_9$), has high affinity for the CB1 receptor ($K_i = 14 \pm 1$ nM); however, the 2-methylindole analog, JWH-242 (**30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_4H_9$), has significantly lower CB1 receptor affinity ($K_i = 42 \pm 9$ nM). The CB2 receptor affinities of the 4-butyl-1-naphthoylindoles are not nearly as diverse as the CB1 affinities. The 1-propyl analogs, JWH-239 and JWH-241 242 (**30**, $R' = C_3H_7$, $= H$ or CH_3, $R'' = C_4H_9$), have $K_i = 52 \pm 6$ nM and $K_i = 49 \pm 7$ nM, respectively. The CB2 receptor affinities for the 1-pentyl compounds, JWH-240 and JWH-242 (**30**, $R = C_5H_{11}$, $R' = H$ or CH_3, $R'' = C_4H_9$), are essentially identical with $K_i = 7.2 \pm 13$ nM for JWH-240 and $K_i = 6.5 \pm 0.3$ nM for JWH-242.

The CB1 receptor affinities for 7-ethyl analogs **31** ($R = C_5H_{11}$, $R' = H$ or CH_3) (Table 3) are similar to that of 1-propyl-2-methyl-3-(7-methyl-1-naphthoyl)indole, JWH-046 (**27**, $R = C_3H_7$). However, in contrast to JWH-046 (**27**, $R = C_3H_7$), which has high affinity for the CB2 receptor ($K_i = 16 \pm 5$ nM) and very little affinity for the CB1 receptor ($K_i = 343 \pm 38$ nM), the corresponding 7-ethyl analog (JWH-239, **31**, $R = C_5H_{11}$, $R' = H$ or CH_3) has little affinity for either receptor (Fig. 8).

In addition to the effect upon CB1 and CB2 receptor affinities of small alkyl groups, the effect of a 4-fluoro, chloro, or bromo substituent upon the receptor affinities of 3-(1-naphthoyl)indoles has been explored (58). The CB1 and CB2 receptor affinities of these compounds (**30**, $R = C_3H_7$ or C_5H_{11}, $R' = H$ or CH_3, $R'' = F$, Cl, Br) are summarized in Table 5. The CB1 receptor affinities of these cannabimimetic indoles exhibit the usual trends in that the *N*-propyl analogs have significantly less affinity for the receptor than the *N*-pentyl compounds and the 2-methylindoles have less affinity for the CB1 receptor than the unsubstituted compounds. The *N*-pentyl compounds have uniformly high affinity for the CB1 receptor ($K_i = 1.2 \pm 0.1$–14 ± 0.7 nM) with relatively little variation in affinity as a function of the halogen. The CB1 receptor affinities of the *N*-propyl



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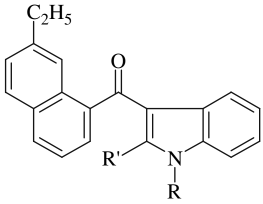

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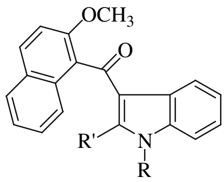

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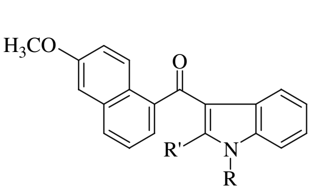

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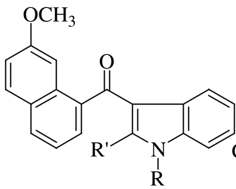

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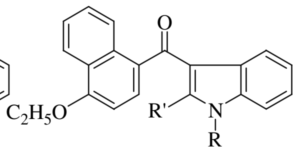

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Fig. 8 Structures of cannabimimetic *N*-alkylindoles with substituted naphthoyl systems. In all cases, $R = C_3H_7$ or C_5H_{11}

analogs range from $K_i = 93 \pm 8$–530 ± 37 nM. In this case the 4-fluoro compounds (**30**, $R = C_3H_7$, $R' = H$ or CH_3, $R'' = F$) have somewhat lower affinity for the CB1 receptor than the corresponding 4-chloro or 4-bromo analogs (**30**, $R = C_3H_7$, $R' = H$ or CH_3, $R'' = Cl$ or Br).

In general the CB2 receptor affinities of the 3-(4-halo-1-naphthoyl)indoles (**30**, $R = C_3H_7$ or C_5H_{11}, $R' = H$ or CH_3, $R'' = F$, Cl or Br) follow the same trends as the CB1 receptor affinities. That is, the *N*-propyl compounds have lower CB2 receptor affinities than the *N*-pentyl analogs, but there is relatively little effect as a function of the substitution at C-2 of the indole nucleus. An exception is in the case of 2-methyl-1-pentyl-3-(4-bromo-1-naphthoyl)indole (JWH-394, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = Br$) which has $K_i = 14 \pm 0.2$ nM at the CB2 receptor, while 1-pentyl-3-(4-bromo-1-naphthoyl)indole (JWH-387, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = Br$) has $K_i = 1.1 \pm 0.1$ nM. Most of these 4-halo naphthoylindoles have from slight-to-moderate selectivity for the CB2 receptor, however, two, 2-methyl-1-pentyl-3-(4-bromo-1-naphthoyl)indole (JWH-394, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = Br$) and 1-pentyl-3-(4-chloro-1-naphthoyl)indole (JWH-398, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = Cl$) have fivefold and very slight selectivity, respectively, for the CB1 receptor.

Both the Sterling and Clemson groups had noted previously that a 4-methoxy-1-naphthoyl substituent enhances CB1 receptor affinity, but virtually nothing was known concerning the effect of a methoxy group in other positions of the naphthoyl moiety [32, 56]. In order to gain insight into the

effect of methoxy groups upon both CB1 and CB2 receptor affinities as well as to probe the effect of a methoxy substituent at other positions of the naphthalene ring, 1-propyl and 1-pentyl-3-(2-methoxy-1-naphthoyl) (**32**), 3-(6-methoxy-1-naphthoyl) (**33**), and 3-(7-methoxy-1-naphthoyl)indoles (**34**), in all cases, $R = C_3H_7$ or C_5H_{11} and $R' = H$ or CH_3 were synthesized and their affinities for the CB1 and CB2 receptor affinities were determined [55]. These receptor affinities are summarized in Table 6.

The 3-(2-methoxy-1-naphthoyl)indoles (**32**, JWH-265 to JWH-268) have very little affinity for the CB1 receptor, with $K_i \geq 380$ nM. However, two of these compounds, JWH-267 (**32**, $R = C_5H_{11}$, $R' = H$) and JWH-268 (**32**, $R = C_5H_{11}$, $R' = CH_3$) have high affinity for the CB2 receptor ($K_i = 7.2 \pm 0.14$ nM and $K_i = 40 \pm 0.6$ nM, respectively). 1-Pentyl-3-(2-methoxy-1-naphthoyl)indole, JWH-267, is a highly selective ligand for the CB2 receptor, with greater than 50-fold selectivity relative to the CB1 receptor.

With the exception of 1-pentyl-3-(6-methoxy-1-naphthoyl)indole (JWH-166, **33**, $R = C_5H_{11}$, $R' = H$) with $K_i = 44 \pm 10$ nM, none of the 3-(6-methoxy-1-naphthoyl)indoles (**33**) have significant affinity for the CB1 receptor. All the other three compounds in this series have $K_i \geq 250$ nM. However, the compounds in this series have from modest to very high affinity for the CB2 receptor. One of them, JWH-151 (**33**, $R = C_3H_7$, $R' = CH_3$) is a highly selective ligand for the CB2 receptor with $K_i = 30 \pm 1.1$ nM and $K_i \geq 10{,}000$ nM at the CB1 receptor.

The 1-pentyl-3-(7-methoxy-1-naphthoyl)indoles (**34**, $R = C_5H_{11}$, $R' = H$ and CH_3) both have high affinity for the CB1 receptor. In particular, 1-pentyl-3-(7-methoxy-1-naphthoyl)indole (JWH-164, (**34**, $R = C_5H_{11}$, $R' = H$) has $K_i = 6.6 \pm$

Table 6 CB1 and CB2 receptor affinities of 3-(alkoxy-1-naphthoyl)indoles [55]

	K_i (nM)	
Compound	CB1	CB2
32, $R = C_3H_7$, $R' = H$, JWH-265	3788 ± 323	80 ± 13
32, $R = C_3H_7$, $R' = CH_3$, JWH-266	>10,000	455 ± 55
32, $R = C_5H_{11}$, $R' = H$, JWH-267	381 ± 16	7.2 ± 0.14
32, $R = C_5H_{11}$, $R' = CH_3$, JWH-268	1379 ± 193	40 ± 0.6
33, $R = C_3H_7$, $R' = H$, JWH-163	2358 ± 215	138 ± 12
33, $R = C_3H_7$, $R' = CH_3$, JWH-151	>10,000	30 ± 1.1
33, $R = C_5H_{11}$, $R' = H$, JWH-166	44 ± 10	1.9 ± 0.08
33, $R = C_5H_{11}$, $R' = CH_3$, JWH-153	250 ± 24	11 ± 0.5
34, $R = C_3H_7$, $R' = H$, JWH-165	204 ± 26	71 ± 8
34, $R = C_3H_7$, $R' = CH_3$, JWH-160	1568 ± 201	441 ± 110
34, $R = C_5H_{11}$, $R' = H$, JWH-164	6.6 ± 0.7	6.9 ± 0.2
34, $R = C_5H_{11}$, $R' = CH_3$, JWH-159	45 ± 1	10.4 ± 1.4
35, $R = C_3H_7$, $R' = H$, JWH-259	220 ± 29	74 ± 7
35, $R = C_3H_7$, $R' = CH_3$, JWH-261	767 ± 105	221 ± 14
35, $R = C_5H_{11}$, $R' = H$, JWH-258	4.6 ± 0.6	10.5 ± 1.3
35, $R = C_5H_{11}$, $R' = CH_3$, JWH-260	29 ± 0.4	25 ± 1.9

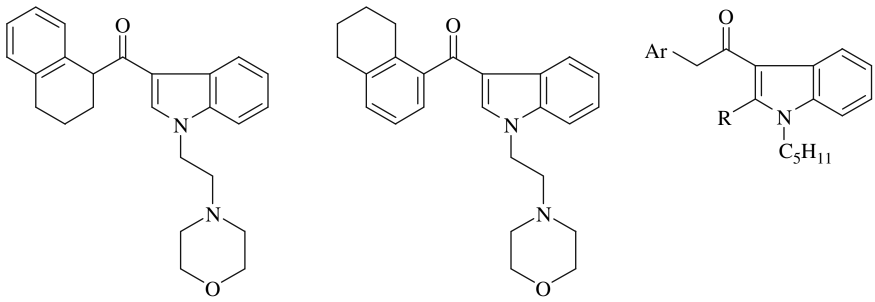
0.7 nM, while the 2-methyl analog (JWH-159, (**34**, $R = C_5H_{11}$, $R' = CH_3$) has somewhat lower affinity with $K_i = 45 \pm 1$ nM. The *N*-propyl compounds, JWH-165 and JWH-160 (**34**, $R = C_3H_7$, $R' = H$ and CH_3), have little affinity for the CB1 receptor with $K_i = 200$ nM.

In the 4-alkyl-1-naphthoyl series (**30**) both 4-ethyl and 4-propyl substituents provide very high CB1 receptor affinities. In order to investigate the effect upon CB1 and CB2 receptor affinities of a 4-alkoxy substituent analogous to a 4-propyl group, a series of 3-(4-ethoxy-1-napthoyl)indoles (**35**, $R = C_3H_7$ and C_5H_{11}, $R' = H$ and CH_3) was prepared and their CB1 and CB2 receptor affinities were determined (Table 6). Indoles **35** have less affinity for the CB1 receptor than the corresponding methoxy analogs (**28** and **29**, Table 6), however the affinities follow the usual trend in that the *N*-propyl indoles (JWH-259, **35**, $R = C_3H_7$, $R' = H$ and JWH-261, **35**, $R = C_3H_7$, $R' = CH_3$) have significantly less CB1 receptor affinity than the *N*-pentyl compounds. Neither JWH-259 nor JWH-261 has a CB1 receptor affinity better than 220 nM. 1-Pentyl-3-(4-ethoxy-1-naphthoyl)indole (JWH-258, **35**, $R = C_5H_{11}$, $R' = H$) has very high affinity for the CB1 receptor with $K_i = 4.6 \pm 0.6$ nM, somewhat less than that for the 4-methoxy analog (JWH-081, **28**, $R = C_5H_{11}$, $K_i = 1.2 \pm 0.1$ nM). The 2-methyl analog (JWH-260, **47**, $R = C_5H_{11}$) has $K_i = 29 \pm 0.4$ nM, which is considerably less than that of the 2-methyl-1-pentyl-3-(4-methoxy-1-naphthoyl)indole (JWH-098, **29**, $R = C_5H_{11}$, $K_i = 4.5 \pm 0.1$ nM).

This study of cannabimimetic indoles led to the discovery of three new highly selective ligands for the CB2 receptor [55]. These compounds are JWH-120, 1-propyl-3-(4-methyl-1-naphthoyl)indole, (**30**, $R = C_3H_7$, $R' = H$, $R'' = CH_3$), 173-fold selective; 1-pentyl-3-(2-methoxy-1-naphthoyl)indole, JWH-267 (**32**, $R = C_5H_{11}$, $R' = H$), 53-fold selective; and 1-propyl-2-methyl-3-(6-methoxy-1-naphthoyl)indole, JWH-151 (**33**, $R = C_3H_7$, $R' = CH_3$), which is >333-fold selective. The efficacy of these compounds was evaluated by their ability to stimulate GTPγS binding. In addition to JWH-120, JWH-151, and JWH-267 the [^{35}S]GTPγS binding for JWH-015, 1-propyl-2-methyl-3-(1-naphthoyl)indole (**6**), the lead compound for the search for CB2-selective cannabimimetic indoles was determined.

These compounds are all potent in the [^{35}S]GTPγS assay with EC_{50} values from 5.1 ± 1.0 nM for JWH-120 (**30**, $R = C_3H_7$, $R' = H$, $R'' = CH_3$) to 17.7 ± 1.0 nM for JWH-015 (**6**). One of these CB2-selective receptor ligands, 1-propyl-2-methyl-3-(6-methoxy-1-naphthoyl)indole, JWH-151 (**33**, $R = C_3H_7$, $R' = CH_3$) is highly efficacious with an E_{max} of $108.5 \pm 13.0\%$ relative to CP-55,940. The other compounds, 1-propyl-2-methyl-3-(1-naphthoyl)indole, JWH-015 (**6**); 1-propyl-3-(4-methyl-1-naphthoyl)indole, JWH-120 (**30**, $R = C_3H_7$, $R' = H$, $R'' = CH_3$); and 1-pentyl-3-(2-methoxy-1-naphthoyl)indole, JWH-267 267 (**32**, $R = C_5H_{11}$, $R' = H$), are partial agonists relative to CP-55,940 with E_{max} values from $65.7 \pm 6.4\%$ (JWH-015) to $78.1 \pm 10.7\%$ (JWH-120).

Among the compounds included in the Sterling study of aminoalkylindoles were two partially reduced derivatives of 3-(1-naphthoyl)indole **9** (Fig. 9) [32]. One of these, 1,2,3,4-tetrahydronaphthalene derivative **36** has moderate affinity



36 **37** **38** R = H or CH_3

Fig. 9 Structures of Sterling partially reduced aminoalkylindoles and 3-phenylacetylindoles

for the CB1 receptor with $IC_{50} \pm 38 \pm 2$ nM, while the other, 5,6,7,8-tetrahydro compound **37** has considerably lower affinity (97% displacement of WIN 55,212-2 at 1000 nM). The fully aromatic analog, indole (**9**) has high CB1 receptor affinity with $IC_{50} \pm 7.8 \pm 0.3$ nM. It was suggested that these compounds were more potent than the 3-benzoylindole analogs due to the presence of a bicyclic system at the 3-position of the indole, rather than specific aromatic interactions. However, based upon subsequent data, which indicates that cannabimimetic indoles interact with the CB1 receptor primarily by aromatic–stacking interactions [17, 57], Huffman et al. synthesized a series of 1-pentyl-3-phenylactylindoles (**38**) [59] in which the aryl group of the phenylacetyl moiety is in a position similar to that of 1,2,3,4-tetrahydronaphthoyl indole **36**. The affinities of these 3-phenylacetylindoles for the CB1 and CB2 receptors are summarized in Table 7. In common with other cannabimimetic indoles, the receptor affinities summarized in Table 7 indicate that in general the 2-methylindoles have lower affinity for the CB1 receptor than the 2-unsubstituted analogs [13, 32, 55, 56]. The compounds with an unsubstituted phenylacetyl group (JWH-167 and JWH-205) have modest affinities ($K_i = 90 \pm 17$ nM and 124 ± 23 nM, respectively) for the CB1 receptor, while the 4-substituted analogs (JWH-208, JWH-209, JWH-201, JWH-202, JWH-313, JWH-316, JWH-206, JWH-207, JWH-248, and JWH-304) have uniformly low CB1 receptor affinity ($K_i = 179$–3363 nM) [59].

The 3-(2-substituted phenylacetyl)indoles have from good-to-high affinity for the CB1 receptor. The highest affinity compounds are 1-pentyl-3-(2-chlorophenylacetyl)indole (JWH-203, **38**, R = H, Ar = 2-chlorophenyl) with $K_i = 8.0 \pm 0.9$ nM and 1-pentyl-3-(2-bromophenylacetyl)indole (JWH-249, **38**, R = H, Ar = 2-bromophenyl) $K_i = 8.4 \pm 1.8$ nM. 1-Pentyl-2-methyl-3-(2-methoxyphenylacetyl)indole (JWH-306, **38**, R = CH_3, Ar = 2-methoxyphenyl); the 1-pentyl-3-(2-fluorophenylacetyl)indoles (JWH-311, **38**, R = H, R′ = 2-fluorophenyl and JWH-314, **38**, R = CH_3, Ar = 2-fluorophenyl); and

Table 7 CB1 and CB2 receptor affinities of 1-pentyl-3-phenylacetylindoles (**38**) [59]

Compound	K_i (nM)	
	CB1	CB2
R = H, Ar = Phenylacetyl, JWH-167	64 ± 17	190 ± 14
R = CH_3, Ar = Phenylacetyl, JWH-205	124 ± 23	180 ± 9
R = H, Ar = 2-Methylphenylacetyl, JWH-251	29 ± 3	146 ± 36
R = CH_3, Ar = 2-Methylphenylacetyl, JWH-252	23 ± 3	19 ± 1
R = H, Ar = 4-Methylphenylacetyl, JWH-208	179 ± 7	570 ± 127
R = CH_3, Ar = 4-Methylphenylacetyl, JWH-209	746 ± 49	1353 ± 270
R = H, Ar = 2-Methoxyphenylacetyl, JWH-250	11 ± 2	33 ± 2
R = CH_3, Ar = 2-Methoxyphenylacetyl, JWH-306	25 ± 1	82 ± 11
R = H, Ar = 3-Methoxyphenylacetyl, JWH-302	17 ± 2	89 ± 15
R = CH_3, Ar = 3-Methoxyphenylacetyl, JWH-253	62 ± 10	84 ± 12
R = H, Ar = 4-Methoxyphenylacetyl, JWH-201	1064 ± 21	444 ± 14
R = CH_3, Ar = 4-Methoxyphenylacetyl, JWH-202	1678 ± 63	645 ± 6
R = H, Ar = 2-Fluorophenylacetyl, JWH-311	23 ± 2	39 ± 3
R = CH_3, Ar = 2-Fluorophenylacetyl, JWH-314	39 ± 2	76 ± 4
R = H, Ar = 3-Fluorophenylacetyl, JWH-312	72 ± 7	91 ± 20
R = CH_3, Ar = 3-Fluorophenylacetyl, JWH-315	430 ± 24	182 ± 23
R = H, Ar = 4-Fluorophenylacetyl, JWH-313	422 ± 19	365 ± 92
R = CH_3, Ar = 4-Fluorophenylacetyl, JWH-316	2862 ± 670	781 ± 105
R = H, Ar = 2-Chlorophenylacetyl, JWH-203	8.0 ± 0.9	7.0 ± 1.3
R = CH_3, Ar = 2-Chlorophenylacetyl, JWH-204	13 ± 1	25 ± 1
R = H, Ar = 3-Chlorophenylacetyl, JWH-237	38 ± 10	106 ± 2
R = CH_3, Ar = 3-Chlorophenylacetyl, JWH-303	117 ± 10	138 ± 12
R = H, Ar = 4-Chlorophenylacetyl, JWH-206	389 ± 25	498 ± 37
R = CH_3, Ar = 4-Chlorophenylacetyl, JWH-207	1598 ± 134	3723 ± 10
R = H, Ar = 2-Bromophenylacetyl, JWH-249	8.4 ± 1.8	20 ± 2
R = CH_3, Ar = 2-Bromophenylacetyl, JWH-305	15 ± 1.8	29 ± 5
R = H, Ar = 4-Bromophenylacetyl, JWH-248	1028 ± 39	657 ± 19
R = CH_3, Ar = 4-Bromophenylacetyl, JWH-304	3363 ± 332	2679 ± 688

the 1-pentyl-3-(2-methylphenylacetyl)indoles (JWH-251, **38**, R = H, Ar = 2-methylphenyl and JWH-252, **38**, R = CH_3, Ar = 2-methylphenyl) have the lowest affinities of this group of compounds with K_i = 23–39 nM. The other 3-(2-substituted phenylacetyl)indoles, JWH-204 (**38**, R = CH_3, Ar = 2-chlorophenyl), JWH-305 (**38**, R = CH_3, Ar = 2-bromophenyl) and JWH-250 (**38**, R = H, Ar = 2-methoxyphenyl) have K_i = 11–15 nM.

Those compounds with a 3-substituted phenylacetyl group have CB1 receptor affinities intermediate between those of the 2- and 4-substituted analogs. In particular, 1-pentyl-3-(3-methoxyphenylacetyl)indole (JWH-302, **38**, R = H, Ar = 3-methoxyphenyl, K_i = 17 ± 2 nM) and 1-pentyl-3-(3-chlorophenylacetyl)indole (JWH-237, **38**, R = H, AR = 3-chlorophenyl, K_i = 38 ± 10 nM) have quite high affinity for the CB1 receptor. Both 1-pentyl-3-(3-fluorophenylacetyl)indole (JWH-312, **38**, R = H, Ar = 3-fluorophenyl) and the corresponding 2-methylindole (JWH-315, **38**, R = CH_3, Ar = 2-fluorophenyl) have modest and little affinity, respectively, for the CB1 receptor.

The CB2 receptor affinities of this class of cannabimimetic indoles tend to follow the same trend as their CB1 affinities (Table 7). Specifically, the 2-substituted phenylacetyl compounds have the greatest affinity, followed by the 3-substituted analogs. The 3-(4-substituted phenylacetyl)indoles have little affinity for the CB2 receptor, and most of the 2-methylindoles have lower CB2 receptor affinities than the unsubstituted analogs. However, in contrast to the usual cannabimimetic indole SAR, 2-methyl-1-pentyl-3-(2-methylphenylacetyl)indole (JWH-252, **38**, R = CH_3, Ar = 2-methylphenyl $K_i = 19 \pm 1$ nM) has more than sevenfold greater affinity for the CB2 receptor than the unsubstituted compound (JWH-251, **38**, R = H, Ar = 2-methylphenyl $K_i = 146 \pm 36$ nM).

Most cannabimimetic indoles show selectivity for the CB2 receptor [14, 56, 57], however, two of these phenylacetylindoles show fivefold selectivity for the CB1 receptor. 1-Pentyl-3-(2-methylphenylacetyl)indole, JWH-251 (**38**, R = H, AR = 2-methylphenyl), has good affinity for the CB1 receptor ($K_i = 29 \pm 3$ nM) with quite modest affinity for the CB2 receptor ($K_i = 146 \pm 36$ nM), while 1-pentyl-3-(3-methoxyphenylacetyl)indole, JWH-302 (**38**, R = H, Ar = 3-methoxyphenyl), also has good affinity ($K_i = 17 \pm 2$ nM) for the CB1 receptor, and fair affinity for the CB2 receptor ($K_i = 89 \pm 15$ nM). To evaluate the efficacy of these compounds, their ability to stimulate [^{35}S]GTPγS binding at CB1 and CB2 was determined. Both compounds stimulate GTPγS binding at CB1 with approximately equal values of EC_{50} (29 nM) and are high efficacy agonists with E_{max} of greater than 90%. Although the affinities of these compounds at CB2 are approximately one-fifth of that of their affinities for the CB1 receptor, both significantly stimulate GTPγS binding at the CB2 receptor. Surprisingly, their potencies for CB2 receptor activation were similar to that seen with CB1; for JWH-251 at CB2, $EC_{50} \pm 8.3 \pm 0.8$ nM and for JWH-302, $EC_{50} \pm 24.4 \pm 6.9$ nM. However, both compounds are partial agonists at the CB2 receptor with E_{max} values of less than 50% [59].

3.3 Structure–Activity Relationships

In the aminoalkylindole series, the Sterling group noted that a morpholinoethyl or piperidinoethyl nitrogen substituent provided compounds with the highest affinity for the CB1 receptor. Subsequent work revealed that an *N*-methyl-2-piperidinylmethyl substituent, as in compound **19**, provided even higher affinity for the CB1 receptor [37]. For compounds with this *N*-aminoalkyl substituent the *R*-configuration provides considerably greater affinity for the CB1 receptor in every case reported to date [41, 48]. For the cannabimimetic *N*-alkyl indoles, CB1 receptor affinity is greatest with an *N*-pentyl group and decreases with decreasing or increasing length of the alkyl chain; however, the *N*-butyl and *N*-hexyl compounds usually have significant affinity for the CB1 receptor [12, 13, 56].

It was noted in the early work of the Sterling group that substitution at C-2 of the indole nucleus by a methyl group slightly attenuated CB1 receptor affinity relative to the unsubstituted congener [11]. This observation was confirmed in subsequent studies by the Sterling group and others [13, 32, 55–59]. The presence of a group larger than methyl at C-2 greatly reduces CB1 receptor affinity [32, 57].

In terms of substitution at C-3 of the indole nucleus, it was observed at an early stage that a bicyclic aroyl substituent, such as 1-naphthoyl, provides greater CB1 receptor affinity than a benzoyl or substituted benzoyl group [11, 32]. The Sterling group reported limited data regarding substitution on a 3-(1-naphthoyl) substituent, but did observe that relatively small electron-donating groups such as methyl or methoxyl in the 4-position enhanced CB1 receptor affinity [32]. More recently the Clemson group has explored the effect on both CB1 and CB2 receptor affinity of 4- and 7-alkyl, 2-, 4-, 6- and 7-methoxy and 4-halo-3-(1-naphthoyl)indoles [55, 56, 58]. These studies were limited to the *N*-propyl and *N*-pentyl indoles.

The 1-pentyl series of cannabimimetic indoles has been explored extensively and provides several structural criteria for developing SAR at the CB1 receptor. As noted above, CB1 receptor affinities are attenuated slightly by the presence of a methyl group at the 2-position of the indole. With the exception of the 1-pentyl-3-(2-methoxy-1-naphthoyl)indoles (JWH-267, **32**, $R = C_5H_{11}$, $R' = H$, $K_i = 381 \pm 16$ nM and JWH-268, **32**, $R = C_5H_{11}$, $R' = CH_3$, $K_i = 1379 \pm 193$ nM) and 1-pentyl-2-methyl-3-(6-methoxy-1-naphthoyl)indole (JWH-153, **33**, $R = C_5H_{11}$, $R' = CH_3$, $K_i = 250 \pm 24$ nM) all of the compounds in this series have high affinity for the CB1 receptor with $K_i \leq 45$ nM. Replacing the hydrogen at C-4 of the naphthalene moiety with a methyl (JWH-122, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = CH_3$, $K_i = 0.69 \pm 0.5$ nM, JWH-149, **30**, $R = C_5H_{11}$, R', $R'' = CH_3$, $K_i = 5.0 \pm 2.1$ nM); ethyl (JWH-210, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_2H_5$, $K_i = 0.46 \pm 0.03$ nM, JWH-213, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_2H_5$, $K_i = 1.5 \pm 0.2$ nM); or propyl (JWH-182, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_3H_7$, $K_i = 0.65 \pm 0.03$ nM, JWH-181, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_3H_7$, $K_i = 1.3 \pm 0.1$ nM) group leads to a considerable increase in CB1 receptor affinity; however a butyl group at C-4 (JWH-240, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_4H_9$, $K_i = 14 \pm 1$ nM, JWH-242, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_4H_9$, $K_i = 42 \pm 9$ nM) results in a marked decrease in CB1 receptor affinity [56]. Neither a 7-methyl-1-napthoyl (JWH-048, **27**, $R = C_5H_{11}$) nor a 7-ethyl-1-naphthoyl (JWH-234, **31**, $R = C_5H_{11}$, $R' = H$, JWH-262, **31**, $R = C_5H_{11}$, $R' = CH_3$) substituent has a significant effect on affinity for the CB1 receptor [55, 56].

In the *N*-propyl 2-, 4-, 6-, and 7-methoxynaphthoyl series (Table 6) the relative magnitudes of the CB1 receptor affinities for the indoles parallel those of the *N*-pentyl analogs. However, the compounds in this series have little affinity for the CB1 receptor with $K_1 \pm 204$ nM to $K_i \geq 10{,}000$ nM. An exception is 1-propyl-3-(4-methoxy-1-naphthoyl)indole (JWH-079, **28**, $R = C_3H_7$), which has moderate affinity for the CB1 receptor with $K_i \geq 63 \pm 3$ nM.

Even with an *N*-pentyl group, a 2-methoxy-1-naphthoyl substituent in JWH-267 (**32**, $R = C_5H_{11}$, $R' = H$) and JWH-268 (**32**, $R = C_5H_{11}$, $R' = CH_3$) effectively destroys affinity for the CB1 receptor while a 4-methoxy group (JWH-081, **28**, $R = C_5H_{11}$, $R' = H$, JWH-098, **28**, $R = C_5H_{11}$, $R' = CH_3$) slightly increases affinity relative to the unsubstituted analogs (JWH-018, **25**, $R = C_5H_{11}$, $R' = H$ and JWH-007, **5**). Replacing the 4-methoxy group with a 4-ethoxy (JWH-258, **35**, $R = C_5H_{11}$, $R' = H$, JWH-260, **35**, $R = C_5H_{11}$, $R' = CH_3$) slightly diminishes CB1 receptor affinity. A 6-methoxy-1-naphthoyl substituent decreases affinity for the CB1 receptor in 1-pentyl-3-(6-methoxy-1-naphthoyl)indole (JWH-166, **33**, $R = C_5H_{11}$, $R' = H$, $K_i = 44 \pm 10$ nM) while the 2-methyl analog (JWH-153, **33**, $R = C_5H_{11}$, $R' = CH_3$) has little affinity. In contrast, the 7-methoxy analogs (JWH-164, **34**, $R = C_5H_{11}$, $R' = H$ and JWH-159, **34**, $R = C_5H_{11}$, $R' = CH_3$) have CB1 receptor affinities comparable to those of the 4-ethoxy compounds [55]. A 4-fluoro-, chloro-, or bromo-1-naphthoyl substituent has an effect upon CB1 receptor affinity that is qualitatively similar to that of a 4-methyl or 4-methoxy substituent. There is relatively little difference in this effect as a function of the halogen substituent [58].

In common with other cannabimimetic indoles, the *N*-propyl analogs have significantly less affinity for the CB1 receptor than the corresponding *N*-pentyl compounds. Although an indole 2-methyl group usually somewhat attenuates CB1 receptor affinity the situation is reversed for several of the *N*-propyl indoles. Most notably, for the compounds with an unsubstituted naphthoyl group, 1-propyl-2-methyl-3-(1-naphthoyl)indole (JWH-015, **6**) and 1-propyl -3-(1-naphthoyl)indole (JWH-072 (**26**, $R = C_3H_7$) and their 4-methyl-1-naphthoyl analogs (JWH-120, **30**, $R = C_3H_7$, $R' = H$, $R'' = CH_3$ and JWH-148, **30**, $R = C_3H_7$, $R' = CH_3$, $R'' = CH_3$) the 2-methyl compounds have CB1 receptor affinities that are nearly 10-fold greater than the unsubstituted compounds (Tables 3 and 4). In the case of the 1-propyl-3-(4-butyl-1-naphthoyl)indoles (JWH-239, **30**, $R = C_3H_7$, $R' = H$, $R'' = C_4H_9$, and JWH-241, **30**, $R = C_3H_7$, $R' = CH_3$, $R'' = C_4H_9$) the 2-methyl analog (JWH-241) has only slightly more than twofold less affinity for the CB1 receptor than JWH-239. Of the *N*-propyl 4- or 7-alkyl-1-naphthoyl analogs only the 4-ethyl-(JWH-211, **30**, $R = C_3H_7$, $R' = CH_3$, $R'' = C_2H_5$ and JWH-212, **30**, $R = C_3H_7$, $R' = H$, $R'' = C_2H_5$) and 4-propyl-1-naphthoylindoles (JWH-180, **30**, $R = C_3H_7$, $R' = H$, $R'' = C_3H_7$, and JWH-189, **30**, $R = C_3H_7$, $R' = CH_3$, $R'' = C_3H_7$) have a CB1 receptor affinity less than 100 nM. In the *N*-pentyl series the 4-ethyl-1-naphthoylindoles (JWH-210, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_2H_5$ and JWH-213, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_2H_5$) and 4-propyl-1-naphthoylindoles (JWH-182, **30**, $R = C_5H_{11}$, $R' = H$, $R'' = C_3H_7$ and JWH-181, **30**, $R = C_5H_{11}$, $R' = CH_3$, $R'' = C_3H_7$) all have exceptionally high affinity for the CB1 receptor. The CB1 receptor affinities of these four cannabimimetic indoles vary from $K_i = 46 \pm 0.03$ nM for JWH-210 to $K_i = 1.2 \pm 0.2$ nM for JWH-213 [55].

In general the SAR for the 3-phenylacetylindoles (**38**, Table 7) follow the same trend as the other cannabimimetic indoles in that the 2-methyl analogs (**38**, R = CH_3) have from moderate to significantly lower affinity for the CB1 receptor than the corresponding unsubstituted analogs (**38**, R = H) [59]. The 1-pentyl-3-(2-methylphenylacetyl)indoles (JWH-251 and JWH-252, **38**, R = H or CH_3, AR = 2-methylphenyl) are an exception to this generalization in that both compounds have effectively the same affinity for the CB1 receptor. In this series of cannabimimetic indoles the nature of the aromatic substituent is not as significant as its orientation on the phenyl ring. The 4-substituted compounds (**38**, R = H or CH_3, AR = 4-substituted phenyl) have uniformly poor affinity for the CB1 receptor.

The 3-(2-substituted phenylacetyl)indoles (**38**, R = H or CH_3) have from good-to-high affinity for the CB1 receptor, ranging from 8.0 ± 0.9 nM for 1-pentyl-3-(2-chlorophenylacetyl)indole (JWH-203, **38**, R = H, AR = 2-chlorophenyl) and 8.4 ± 1.8 nM for 1-pentyl-3-(2-bromophenylacetyl)indole (JWH-249, **38**, R = H, AR = 2-bromophenyl) to 39 ± 2 nM for 1-pentyl-2-methyl-3-(2-fluorophenylacetyl)indole (JWH-314, **38**, R = CH_3, AR = 2-fluorophenyl). 1-Pentyl-3-(2-methylphenylacetyl)- (JWH-251, **38**, R = H, AR = 2-methylphenyl and JWH-252, **38**, R = CH_3, AR = 2-methylphenyl) and 1-pentyl-3-(2-methoxyphenylacetyl)indoles (JWH-250, **38**, R = H, AR = 2-methoxyphenyl and JWH-306, **38**, R = CH_3, AR = 2-methoxyphenyl) have CB1 receptor affinities from 11 ± 2 nM (JWH-250) to 29 ± 3 nM (JWH-251). The 2-methyl analogs of these cannabimimetic indoles have slightly lower affinities for the CB1 receptor. The 3-(3-substituted phenylacetyl)indoles have from slightly to significantly lower affinity for the CB1 receptor than the corresponding 2-substituted analogs.

To summarize the SAR at the CB1 receptor for cannabimimetic indoles, receptor affinity is maximized by a pentyl or an *N*-methyl-2-piperidinylmetthyl nitrogen substituent. A morpholinoethyl, piperidinoethyl, butyl or hexyl substituent reduces CB1 receptor affinity somewhat. Other heterocyclic, amino or alkyl substituents in general have a markedly adverse effect upon CB1 receptor affinity. A 2-methyl group appended to the indole nucleus reduces CB1 receptor affinity somewhat relative to the unsubstituted compound, while a larger C-2 substituent considerably attenuates affinity for the CB1 receptor.

The receptor affinities of the 1-pentyl- and 1-propyl-3-(1-naphthoyl)indoles with a 4- and 7-alkyl-1-naphthoyl or 2-, 4-, 6-, 7-methoxy, 4-ethoxynaphthoyl and 4-halo- substituents show that affinity for the CB1 receptor is enhanced considerably by the presence of small alkyl groups (methyl, ethyl, propyl) at C-4. Methyl or ethyl substituents at C-7 of the naphthoyl group have little effect on affinity relative to the unsubstituted compounds. A methoxy or halogen substituent at C-4 enhances CB1 receptor affinity, while a 4-ethoxy substituent has relatively little effect on affinity. 6-Methoxy-1-naphthoylindoles have CB1 receptor affinities that are attenuated relative to their unsubstituted analogs, while a 7-methoxy substituent has little effect upon affinity. A 2-methoxy substituent effectively destroys affinity for the CB1 receptor.

Although a considerable number of cannabimimetic indoles, with both *N*-aminoalkyl and *N*-alkyl substituents have been described and their CB2 receptor affinities have been determined, it is difficult at this point to present comprehensive SAR for this class of cannabinoids at the CB2 receptor. In general, cannabimimetic indoles show selectivity for the CB2 receptor and several of them have the desirable combination of high affinity for the CB2 receptor and slight affinity for the CB1 receptor. However, these selective ligands belong to diverse structural classes of cannabimimetic indoles. Four of these selective indoles are *N*-propyl compounds (JWH-015, JWH-046, JWH-120 and JWH-151) while one is an *N*-pentylindole derivative (JWH-267) [56] and one (AM1241) has an *N*-methyl-2-piperidinylmethyl nitrogen substituent [38]. These compounds are all CB2 receptor agonists or partial agonists and one indole-based CB2 selective inverse agonist (AM630) has been described [33]. AM630 has an *N*-morpholinoethyl substituent and in common with AM-1241 has a substituted 3-benzoyl group appended to the indole nucleus. The five compounds from the Clemson group all have a 3-(1-naphthoyl) or substituted 3-(1-naphthoyl) substituent.

In contrast to their CB1 receptor affinities, the CB2 receptor affinities of the *N*-alkyl indoles described by the Clemson group show comparatively little variation, provided the alkyl group appended to nitrogen contains from three to six carbon atoms [55, 56, 58, 59]. In the 4-alkylnaphthoyl series, none of the compounds have CB2 receptor affinities greater than 52 nM, however 1-propyl-3-(7-methyl-1-naphthoyl)indole (JWH-076) and both 1-propyl-3-(7-ethyl-1-naphthoyl)indoles (JWH-235 and JWH-236) have relatively slight affinity for the CB2 receptor [56]. In the alkoxynaphthoyl series there appears to be no clear pattern and most of these cannabimimetic indoles have good-to-moderate affinity for the CB2 receptor, however in some cases a 1-propyl-2-methylindole substitution pattern leads to attenuated CB2 receptor affinity.

A 2-methyl substituent on the indole nucleus generally leads to a decrease in affinity for either receptor relative to the unsubstituted analog. Similarly, the 1-propylindoles in general have lower affinities at both receptors than the 1-pentyl compounds. 1-Butyl and 1-hexylindoles usually have intermediate affinities for both receptors, however the differences in CB2 receptor affinities as a function of *N*-substitution are less pronounced than is the case with CB1 receptor affinities. Although several highly selective indole-based ligands for the CB2 receptor have been developed, the current knowledge of the SAR at this receptor is such that it is difficult to clearly define the structural criteria for the development of additional selective ligands.

4 Cannabimimetic Pyrroles

The cannabimimetic *N*-alkylindoles prepared by the Clemson group were based on an alignment of WIN-55,212-2 (**4**) and Δ^9-THC (**1**) that assumed that the ketonic carbonyl of **4** corresponded to the phenolic hydroxyl of THC and that

the cyclohexene ring of THC would align with the naphthalene ring of WIN-55,212-2 [12]. This alignment was developed using a computer modeling program, which indicated that the benzenoid ring of the indole nucleus of WIN-55,212-2 was redundant since it did not correspond to any of the atoms of THC. Based upon this observation it appeared possible that pyrrole derivatives (**7**, R = various alkyl groups) would show cannabinoid pharmacology. A series of *N*-substituted 3-(1-naphthoyl)pyrroles (**7**, R = CH_3 to C_7H_{15}) was prepared and their in vitro and in vivo pharmacology were evaluated [13, 15]. The 1-methyl, ethyl, and propyl analogs (JWH-044, **7**, R = CH_3, JWH-045, **7**, R = C_2H_5 and JWH-032, **7**, R = C_3H_7) have no affinity for the CB1 receptor with $K_i \geq 10{,}000$ nM), but show weak in vivo effects in the spontaneous activity and rectal temperature assays. The *N*-propyl analog, JWH-032, also exhibits very weak antinociceptive effects. The *N*-butylpyrrole (JWH-033, **7**, R = C_4H_9) has very little affinity for the CB1 receptor ($K_i = 666 \pm 77$ nM) and is effectively inactive in vivo. 1-Pentyl- 3-(1-naphthoyl)pyrrole (JWH-030, **7**, R = C_5H_{11}) has modest affinity for the CB1 receptor with $K_i = 87 \pm 3$ nM and is quite potent in the spontaneous activity and TF assays, but lacks potency in inducing hypothermia and in the RI protocol. In addition, JWH-030 has very little affinity for the CB2 receptor with $K_i = 320 \pm 127$ nM (Huffman, JW, unpublished work). The *N*-methyl–*N*-propyl pyrrole analogs have no affinity for the CB2 receptor. The 1-hexyl (JWH-031, **7**, R = C_6H_{13}) and 1-heptyl (JWH-036, **7**, R = C_7H_{15}) compounds have little affinity for the CB1 receptor with $K_i = 399 \pm 109$ nM and $K_i = 309 \pm 11$ nM, respectively [15]. These pyrrole derivatives are quite potent in the TF and spontaneous activity assays, but are considerably less potent in the RT and RI procedures. JWH-030 (**7**, R = C_5H_{11}) and JWH-031 (**7**, R = C_6H_{13}) were evaluated in the drug discrimination protocol in rats and both produced dose-dependent substitution for Δ^9-THC [13].

The effects of 1-pentyl-3-(1-naphthoyl)pyrrole (JWH-030, **7**, R = C_5H_{11}) upon the inhibition of the electrically evoked contractions of the MVD were also evaluated and it was found that this compound caused a dose-dependent inhibition of the electrically evoked contractions that were antagonized by the CB1 receptor antagonist, SR-141716A [55]. The combination of CB1 receptor affinity, in vivo pharmacology and inhibition of electrically induced contractions of the mouse vas deferens that were antagonized by SR-141716A all strongly support the hypothesis that *N*-pentyl-3-(1-naphthoyl)pyrrole (JWH-030, **7**, R = C_5H_{11}) is a cannabinoid CB1 receptor agonist. The CB2 receptor affinities of only four of these pyrrole derivatives, JWH-044 (**7**, R = CH_3), JWH-045 (**7**, R = C_2H_5), JWH-032 (**7**, R = C_3H_7), and JWH-030 (**7**, R = C_5H_{11}) have been determined (Huffman, JW, unpublished work). JWH-030 has slight affinity for the CB2 receptor ($K_i = 320 \pm 127$ nM), while the other three compounds have none with $K_i \geq 10{,}000$ nM.

Based upon the current hypothesis that cannabimimetic indoles, and by extending the corresponding pyrroles, interact with the CB1 receptor principally by aromatic stacking [17, 57], it was concluded that an additional aromatic ring appended to the pyrrole nucleus of **7** could provide cannabimimetic

Table 8 Receptor affinities of 2-phenyl-4-(1-naphthoyl)pyrroles (**39**) [60]

	K_i (nM)	
Compound	CB1	CB2
$R = C_3H_7$, JWH-156	404 ± 19	104 ± 18
$R = C_4H_9$, JWH-150	60 ± 1	15 ± 2
$R = C_5H_{11}$, JWH-145	14 ± 2	6.4 ± 0.4
$R = C_6H_{13}$, JWH-147	11 ± 1	7.1 ± 0.2
$R = C_7H_{15}$, JWH-146	21 ± 2	62 ± 5

pyrroles with greater affinity for the CB1 receptor than had been observed previously. To test this hypothesis, a series of 2-phenyl-4-(1-naphthoyl)-*N*–alkyl pyrroles (**39**, $R = C_3H_7$ to C_7H_{15}) was synthesized and their affinities for the CB1 and CB2 receptor were determined (Table 8) [60]. With the exception of the *N*-propyl derivative (JWH-156, **39**, $R = C_3H_7$) these compounds have from moderate-to-high affinity for both the CB1 and CB2 receptors. The *N*-pentyl (JWH-145, **39**, $R = C_5H_{11}$) and *N*-hexyl (JWH-147, **39**, $R = C_6H_{13}$) analogs have high affinity for the CB1 receptor with $K_i = 14 \pm 2$ nM and $K_i = 11 \pm 1$ nM, respectively. Both compounds have slightly higher affinity for the CB2 receptor with $K_i = 6.4 \pm 0.4$ nM and 7.1 ± 0.2 nM.

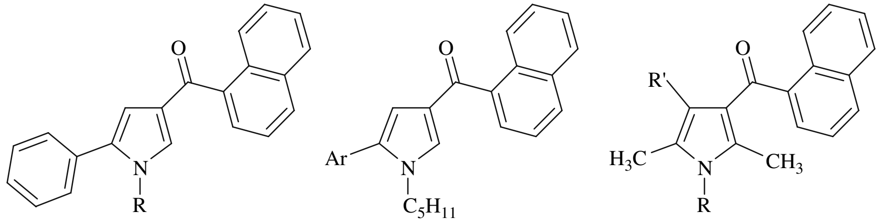
Based upon the high affinity of pyrroles **39**, a series of 21 analogs of JWH-145 (**39**, $R = C_5H_{11}$) with the 2-phenyl substituent replaced by a substituted phenyl group at the 2-position (**40**, Ar = various aryl groups) were synthesized and the affinities for both the CB1 and CB2 receptors were determined (Table 9) (Fig. 10) [60, 61], In general those pyrrole derivatives with relatively small, *ortho* substituents, which are electron releasing inductively or by resonance effects, such as methyl (JWH-370), ethyl (JWH-365), methoxy (JWH-292), or halogen (JWH-307, JWH-369) have high affinity for the CB1 receptor with $K_i = 5.6$–29 nM. Larger *ortho* substituents, such as butyl (JWH-373) or the strongly electron-withdrawing trifluoromethyl group (JWH-392), provide reduced affinity for the CB1 receptor. For JWH-373, $K_i = 60 \pm 3$ nM and for JWH-392, $K_i = 77 \pm 2$ nM. The *meta*-substituted compounds in this series have reduced affinity for the CB1 receptor relative to the *ortho*-substituted analogs and the compounds with *para* substituents in general have relatively modest to very little affinity for the CB1 receptor. An exception is the *para*-butylphenyl analog (JWH-371, **40**, Ar = 4-butylphenyl), which has slightly greater affinity for the CB1 receptor ($K_i = 42 \pm 1$ nM) than the *ortho*-butyl isomer (JWH-373, **40**, Ar = 2-butylphenyl) with $K_1 \pm 60 \pm 3$ nM. One pyrrole derivative with a strongly electron-withdrawing 2-*meta*-nitrophenyl substituent (JWH-293, **40**, Ar = 3-nitrophenyl) was evaluated and found to have modest affinity ($K_i = 100 \pm 5$ nM) for the CB1 receptor.

As is the case with the affinities of the 1-pentyl-2-aryl-4-(1-naphthoyl)pyrroles (**40**) for the CB1 receptor, the affinities of the *ortho*-substituted aryl compounds for the CB2 receptor are uniformly high with $K_i = 3.3$–39 nM. With the exception of the 2-*para*-methoxyphenyl analog (JWH-243, **40**,

Table 9 CB1 and CB2 receptor affinities of 1-pentyl-2-aryl-4-(1-naphthoyl)pyrroles (**40**) [60, 61]

Compound	K_i (nM)	
	CB1	CB2
Ar = 2-Methylphenyl, JWH-370	5.6 ± 0.4	3.4 ± 0.2
Ar = 3-Methylphenyl, JWH-346	67 ± 6	49 ± 7
Ar = 4-Methylphenyl, JWH-244	130 ± 6	15 ± 2
Ar = 2-Ethylphenyl, JWH-365	17 ± 1	39 ± 2
Ar = 4-Ethylphenyl, JWH-364	34 ± 3	23 ± 1
Ar = 2-Butylphenyl, JWH-373	60 ± 3	9.1 ± 0.7
Ar = 4-Butylphenyl, JWH-371	42 ± 1	24 ± 1
Ar = 2-Fluorophenyl, JWH-307	7.7 ± 1.8	7.1 ± 0.2
Ar = 3-Fluorophenyl, JWH-368	16 ± 1	71 ± 0.2
Ar = 4-Fluorophenyl, JWH-308	41 ± 1	62 ± 5
Ar = 2-Chlorophenyl, JWH-369	7.9 ± 0.4	29 ± 0.2
Ar = 3-Chlorophenyl, JWH-246	70 ± 4	18 ± 0.01
Ar = 4-Chlorophenyl, JWH-245	276 ± 4	6.4 ± 0.4
Ar = 2-Methoxyphenyl, JWH-292	29 ± 1	3.3 ± 0.2
Ar = 3-Methoxyphenyl, JWH-367	53 ± 2	33 ± 2
Ar = 4-Methoxyphenyl, JWH-243	285 ± 40	104 ± 18
Ar = 2-Trifluoromethylphenyl, JWH-392	77 ± 2	24 ± 1
Ar = 3-Trifluoromethylphenyl, JWH-363	245 ± 5	20 ± 1
Ar = 4-Trifluoromethylphenyl, JWH-348	218 ± 19	41 ± 4
Ar = 3-Nitrophenyl, JWH-293	100 ± 5	25 ± 2

Ar = 4-methoxyphenyl, K_i = 104 ± 18 nM) and the 2-*para*-fluorophenylpyrrole (JWH-308, **40**, Ar = 4-fluorophenyl, K_i = 71 ± 0.2 nM) all of these 2-arylpyrroles have from high-to-moderate affinity for the CB2 receptor (K_i = 3.3–49 nM). There is considerably less variation in affinity for this receptor as a function of the nature of the aryl group than exists for the affinities of these compounds for the CB1 receptor (Table 9). One of these cannabimimetic pyrroles, 1-pentyl-2-(4-chlorophenyl)-4-(1-naphthoyl)pyrrole (JWH-245, **40**, Ar = 4-chlorophenyl) has 43-fold selectivity for the CB2 receptor with



39 **40** **41**

Fig. 10 Structures of 2-aryl- and 2,5-dimethylpyrroles

Table 10 CB1 and CB2 receptor affinities for 2,5-dimethyl-3-(1-naphthoyl)pyrroles (**41**) and JWH-030 (**7**) [62]

R	R′	EC_{50} (nM)	
		CB1	CB2
C_5H_{11}[a]	H	30.5 ± 4.7	552 ± 314
C_5H_{11}	H	45.3 ± 7.5	9.85 ± 2.1
p-$ClC_6H_4CH_2$	H	83.7 ± 17.8	55.6 ± 26.5
C_5H_{11}	Br	13.3 ± 0.5	6.8 ± 1.0
p-$ClC_6H_4CH_2$	Br	38 ± 7.2	194.5 ± 27.5

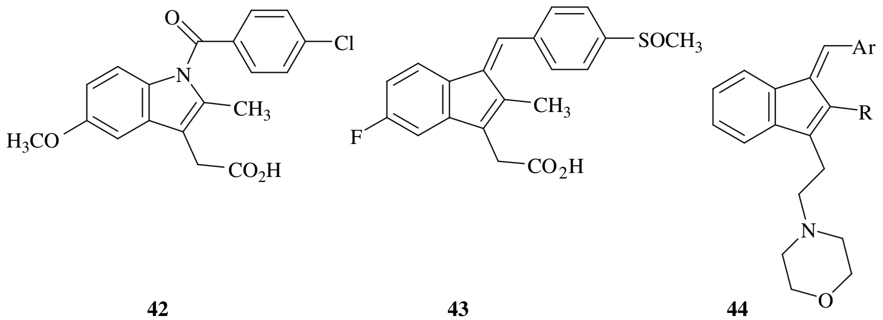
[a] JWH-030

$K_i = 6.4 \pm 0.4$ nM at the CB2 receptor and 276 ± 4 nM at the CB1 receptor. The *meta*-trifluoromethylphenyl compound (JWH-363, **40**, Ar = 3-trifluoromethylphenyl) also exhibits significant, 12-fold, selectivity for the CB2 receptor with $K_i = 20 \pm 1$ nM at CB2 and $K_i = 245 \pm 5$ at the CB1 receptor. None of the other compounds in this series show significant selectivity for either the CB1 or CB2 receptor.

A second group of cannabimimetic pyrroles (**41**) the structures of which were based upon that of 1-pentyl-3-(1-naphthoyl)pyrrole (JWH-030, **7**) was described by Tarzia et al. [62]. These compounds contain 2,5-dimethyl substituents and those with significant affinity for either the CB1 or CB2 receptor include a 3-(1-naphthoyl) substituent with an *N*-pentyl (**41**, R = C_5H_{11}) or *N*-*para*-chlorobenzyl (**41**, R = 4-$ClC_6H_4CH_2$) group with either a hydrogen (**41**, R′ = H) or bromine (**41**, R′ = Br) at C-4 of the pyrrole nucleus. As shown in Table 10, these compounds have affinities for the CB1 receptor in the same range as that of JWH-030 (**7**), however they all have significantly higher affinity for the CB2 receptor than JWH-030. The effect of these four compounds on [^{35}S]GTPγS binding in rat cerebellar membranes was determined and it was found that the two compounds with *N*-pentyl substituents (**41**, R = C_5H_{11}, R′ = H or Br) are full agonists at the CB1 receptor, while the two compounds with an *N*-*p*-chlorobenzyl group (**41**, R = 4-$ClC_6H_4CH_2$, R′ = H or Br) appear to be weak partial agonists [62]. These authors also determined the [^{35}S]GTPγS binding for JWH-030 (**7**) and report an EC_{50} value of 95.5 ± 13.5 nM, which indicates significantly enhanced [^{35}S]GTPγS binding relative to the 2,5-dimethylpyrroles (**41**), however the efficacy of JWH-030 was not reported. Tarzia et al. also prepared a number of other 2,5-dimethylpyrroles, including those with a 3-benzoyl substituent or an *N*-propyl group, however none of these compounds had significant affinity for either the CB1 or CB2 receptor.

5 Cannabimimetic Indenes

During clinical trials of pravadoline (**3**) the Sterling group observed unexpected CNS side effects that ultimately were found to be due to the interaction of paravadoline with the CB1 receptor [11, 30]. In an effort to circumvent these

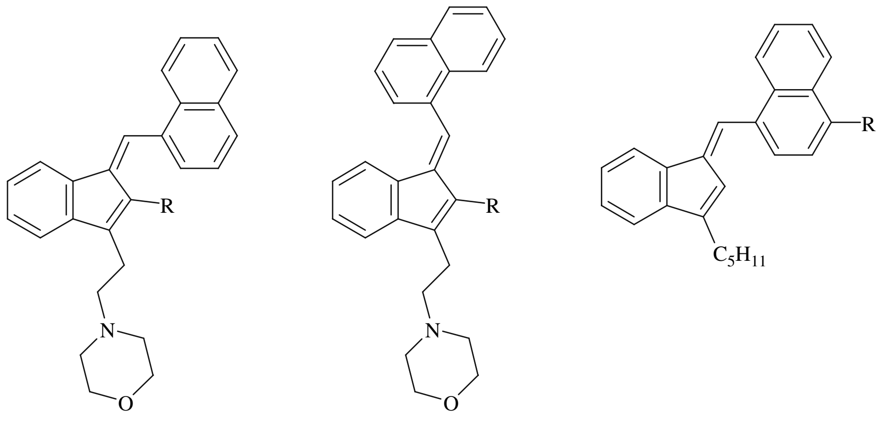


42 43 44

Fig. 11 Structures of indomethacin, sulindac, and the Sterling indenes

CNS effects an indene analog of indomethacin (**42**), sulindac (**43**), was prepared and found to have anti-inflammatory activity comparable to that of indomethacin (Fig. 11). The group at Sterling synthesized a series of eleven 1-[2-(4-morpholino)ethyl]-3-arylidene derivatives of indene (**44**, R = H or CH_3, Ar = aryl), which are indene analogs of pravadoline (**3**) [16]. These compounds were obtained as mixtures of *E*- (depicted in **44**) and *Z*-isomers. In the series in which R = H, the *E*-isomer was the predominant product (95%), and when R = CH_3, a 4:1 mixture of *E*- and *Z*-isomers was obtained. The indene derivatives were evaluated for their ability to displace WIN-55,212-2 (**4**) from a rat brain membrane preparation, and in the inhibition of the electrically stimulated contraction of the mouse vas deferens. The aromatic substituents included several substituted phenyl groups, none of which had significant affinity for the cannabinoid receptor. However, the *E*-naphthylidene analogs **44**, Ar = 1-naphthyl, and 4-methoxy-1-naphthyl, have high affinity for the CB1 receptor (IC_{50} ± 1.0 and 0.9 nM, respectively). The 2-methyl indene (**44**, R = CH_3, 4:1 mixture of *E*- and *Z*-isomers, AR = 1-naphthyl) has significantly lower affinity for the receptor (IC_{50} ± 10.0 nM).

In a nearly simultaneous and independent study Reggio et al. reported cannabimimetic indenes **45** and **46** (R = H or CH_3) (Fig. 12) [17]. These compounds were designed as rigid mimics for the *s*-trans and *s*-cis conformational isomers of cannabimimetic indoles. The *s*-trans conformation of cannabimimetic indoles, in which the 3-aryl substituent is proximate to C-2 of the indole nucleus, is the principal conformation when the C-2 position of the indole is unsubstituted. This conformation is considered to correspond to the *E*-isomer of naphthylidene indenes, **45** (R = H or CH_3). The *s*-cis conformation of the indoles, in which the 3-aryl substituent is near C-4 of the indole nucleus, is the major conformation those indoles with a 2-methyl substituent. This conformation corresponds to the *Z*-isomer of naphthylidene indenes **46** (R = H or CH_3). In contrast to the work of Kumar et al. in which a mixture of the *E*- and *Z*-isomers of the indenes was employed for pharmacological evaluation [16], the compounds described by Reggio et al. were carefully purified and the



45 **46** **47** R = H
48 R = CH_3

Fig. 12 Structures of cannabimimetic indenes

structures were confirmed by NMR techniques [17]. Both *E*-isomers (**45**, R = H or CH_3) have high and nearly identical affinity for both the CB1 and CB2 receptors ($K_i = 2.05–2.89$ nM), however in the inhibition of electrically invoked contractions of the myenteric plexus–longitudinal muscle of the guinea pig small intestine **45**, R = CH_3, was significantly less potent than the analog without the methyl substituent (**45**, R = H). The *Z*-isomers (**46**, R = H or CH_3) have little affinity for the either receptor, with $K_i = 132$ and 1945 nM. For this pair of compounds the analog with the methyl substituent (**46**, R = CH_3) has significantly lower affinity for both receptors ($K_i = 1945 \pm 94$ nM at the CB1 receptor and 658 ± 206 nM at the CB2 receptor) than the unsubstituted compound (**46**, R = H) with $K_i = 148 \pm 29$ nM at the CB1 receptor and 132 ± 46 nM at the CB2 receptor. Since the *E*-isomers are rigid analogs of the *s*-trans conformation of cannabimimetic indoles, the tentative conclusion reached was that the *s*-trans conformation may represent the preferred conformation at the CB1 and CB2 receptors [17].

Experimental data obtained from work with mutant receptors and molecular modeling studies strongly suggest that cannabimimetic indoles interact with the cannabinoid receptors primarily through aromatic stacking [17, 57, 63–65]. The importance of aromatic group orientation in the indene series supports this hypothesis and implies that the indenes interact with the CB1 receptor in the same manner as the cannabimimetic indoles [17]. In order to exclude a possible hydrogen-bonding mechanism of these ligands with the receptor, the Clemson group prepared indenes **47** (JWH-176) and **48** (JWH-220), which have a pentyl group in place of the morpholinoethyl substituent present in indenes **44**, **45,** and

46 ([57] and Huffman, JW; Mabon, R unpublished work). These compounds were obtained as the *E*-isomers and both have high affinity for the CB1 receptor with $K_i = 26 \pm 4$ nM (**47**) and $K_i = 19 \pm 4$ nM (**48**). Indenes **47** and **48** both have somewhat lower affinity for the CB2 receptor with $K_1 = 92 \pm 20$ nM and $K_1 = 41 \pm 4$ nM, respectively. The presence of a 4-methyl substituent on the naphthalene moiety of **48** leads to somewhat enhanced affinity for both receptors as it does in the indole series.

6 Receptor Interactions

6.1 The CB1 Receptor

It was originally assumed that the aminoalkylindoles (AAIs) bind to the CB1 receptor at the same site as traditional cannabinoids, and that a universal cannabinoid pharmacophore could be developed that would accommodate both classes of ligands [12, 13, 32, 66, 67]. Two rather different alignments of WIN-55,212-2 (**4**) and Δ^9-THC (**1**) were proposed; in one, suggested originally by the Sterling group [32], which was later modified by two other groups [66, 67], the naphthalene ring system coincides with the aliphatic side chain of traditional cannabinoids. In the original Sterling model it was suggested that the amino group of the AAIs interacts at the same site as the phenolic hydroxyl of traditional cannabinoids [32]. The modifications retained the alignment of the naphthalene system and the side chain, but proposed that the aminoalkyl group mimicked C-11 of the traditional cannabinoid [66, 67]. This suggested alignment was tested experimentally by the synthesis and pharmacological evaluation of a series of 1-morpholinoethyl-4-alkoxyindoles, but these compounds have low affinity for the CB1 receptor, and were not potent in vivo [66].

A very different alignment was suggested by Huffman et al., who suggested that the naphthoyl carbonyl corresponds to the phenolic hydroxyl of traditional cannabinoids [12, 13]. The 7- and 8-positions of the naphthalene moiety in WIN-55,212-2 were overlaid upon C-9 and C-10 of THC, in which alignment the indole nitrogen corresponds to the first carbon atom of the cannabinoid side chain (C-1′, for numbering, see structures **1** and **4**). The conclusion was reached that if this alignment was correct, the aminoalkyl group was not essential for cannabinoid activity and could be replaced by other substituents. Based upon this design, over 200 1-alkyl-3-(aroyl)indoles, 1-alkylpyrroles, and alkylindenes have been synthesized and their pharmacology evaluated [12, 13, 15, 55–59]. Some of these indole derivatives are very potent cannabinoids, confirming that in contrast to the assertions of the Winthrop group, an aminoalkyl substituent is not essential for cannabinoid activity.

Other attempts to derive a CB1 pharmacophore that would accommodate both traditional cannabinoids, such as Δ^9-THC, and the cannabimimetic indoles were proposed by Shim et al. [38, 51] and by Fichera et al. [68]. Both

the studies by Shim et al. and Fichera et al. employed three-dimensional quantitative structure–activity (3D-QSAR) methods, but they reached considerably different conclusions. Shim et al. considered two conformations of WIN-55,212-2 (**4**), one that they designated the *Z-form* the other the *C-form* [52]. The *Z-form* is the *s*-trans conformation, which, based on Reggio′s indene work, is probably the active conformation [17]. The *Z-form* of WIN-55,212-2 was overlapped with CP-55,244, a very potent tricyclic analog of CP-55,940 in a manner somewhat similar to that suggested by Xie et al. [67]. A 3D-QSAR was constructed by CoMFA to test the validity of this alignment [38, 51]. The alignment suggested by Fichera et al. [68] was essentially that proposed by Huffman et al. in which the C-3 alkyl side chain of the traditional cannabinoids corresponds to the substituent on the nitrogen of the cannabimimetic indole, the acyl carbonyl group of the indole is a surrogate for the phenolic hydroxyl group of the traditional cannabinoid and the indole C-3 aromatic group corresponds to the nonaromatic cyclohexene ring of THC or its analogs [12, 13].

Experiments employing mutant CB1 receptors initially led to the suggestion that cannabimimetic indoles and traditional cannabinoids, such as Δ^9-THC (**1**) bind to different, but partially overlapping sites on the receptor [39, 69]. In particular, it was observed that replacement of a lysine on helix 3 of the CB1 receptor with an alanine greatly attenuated the receptor affinities of the very potent classical cannabinoid HU-210 and CP-55940 (**2**), but only slightly reduced the affinity of WIN-55,212-2 [39]. These mutation data, combined with molecular modeling studies, led to a hypothesis that a hydrogen-bonding interaction of a lysine on the third transmembrane domain of the CB1 receptor is important in the binding of traditional cannabinoids such as Δ^9-THC (**1**), but not cannabimimetic indoles [17, 70]. These data indicated that traditional cannabinoids and cannabimimetic indoles interact with the CB1 receptor in uniquely different ways. It was suggested that these two classes of cannabinoid receptor ligands bind in somewhat different locations on the receptor and that while the hydrogen-bonding interaction with a lysine on helix 3 of the receptor is important for traditional cannabinoids that the binding of cannabimimetic indoles is primarily by aromatic stacking interactions [63, 70–72].

The aromatic stacking hypothesis was supported by the observation that indenes **45** to **48** had high affinity for the CB1 receptor [17, 58, 65]. In particular, indenes **47** and **48** are hydrocarbons that would not be expected to interact with the CB1 receptor by hydrogen bonding. More recent work, which employed the combination of molecular modeling and the single-point mutation of four aromatic amino acids (two phenylalanines and two tryptophans) in the transmembrane helix 3-4-5 region of the CB1 receptor found that these mutations adversely affected the binding of WIN-55,212-2 (**4**) but not CP-55,940 (**2**) [73]. The conclusion was reached that a microdomain of aromatic amino acids comprised of phenylalanine, tryptophan, and tyrosine included in transmembrane helices 3-4-5-6 of the CB1 receptor constitute the binding region for WIN-55,212-2 and presumably other cannabimimetic indoles.

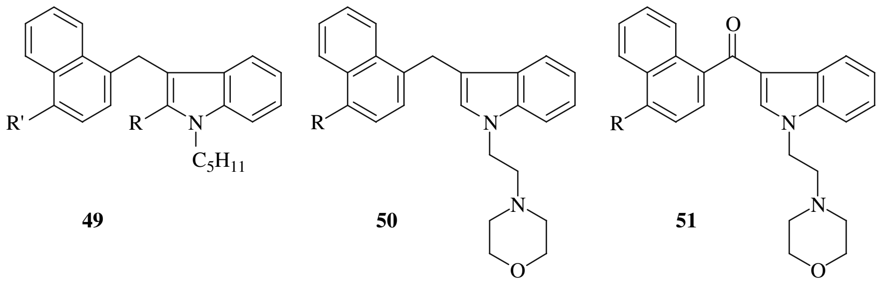


Fig. 13 Structures of 3-indolyl-1-naphthylmethanes and naphthoyl analog

To test the hypothesis that aromatic stacking interactions are important in the interaction of cannabimimetic indoles with the CB1 receptor rather than hydrogen bonding involving the indole carbonyl group, two series of 3-indolyl-1-naphthylmethanes (**49**, R = H or CH_3, R′ = H, CH_3 or OCH_3 and **50**, R = H, CH_3 or OCH_3) were prepared and their affinities for the CB1 receptor were determined (Fig. 13) [57].

The CB1 receptor affinities of indoles **49**, R = H (JWH-175, JWH-184 and JWH-185, R′ = H, CH_3 or OCH_3), which are unsubstituted at the C-2 position of the indole, are essentially identical with K_i = 17–23 nM [57]. These affinities are slightly less than those of the naphthoylindoles with the same substituents at the 4-position of the naphthalene ring, JWH-018 (**5**), JWH-122 (**30**, R = C_5H_{11}, R′ = H, R″ = CH_3), and JWH-081 (**28**, R = C_5H_{11}), which have $K_i = 9 \pm 5$, 0.69 ± 0.05, and 1.2 ± 0.1 nM, respectively. Although the presence of a methyl or methoxy group at the 4-position of the naphthoyl group causes an increase in affinity for naphthoylindoles JWH-122 and JWH-127, in indolylnaphthylmethanes **49** there is little effect upon CB1 receptor affinity as a function of substitution at C-4. In the naphthoylindole series a methyl group at C-2 of the indole causes slight attenuation of CB1 receptor affinity, however indolylnaphthylmethanes **49**, R = CH_3 (JWH-196, JWH-194, and JWH-197) have very much reduced affinity for the CB1 with K_i = 151–323 nM [57].

The Sterling cannabimimetic indoles have an aminoalkyl group as a substituent on the indole nitrogen [10, 11, 32, 37]. It is conceivable that this aminoalkyl group could interact with the CB1 receptor by hydrogen bonding as suggested by Xie et al. [67]. To explore this possibility, aminoalkylindoles **50** (JWH-195, R = H; JWH-192, R = CH_3; JWH-199, R = OCH_3), which lack the carbonyl oxygen, and the corresponding naphthoyl analogs, JWH-200 (**51**, R = H), JWH-193 (**51**, R = CH_3), and JWH-198 (**51**, R = OCH_3), were prepared and their affinities for the CB1 receptor were determined [57]. Although there is little variation in CB1 receptor affinity as a function of substitution at C-4 of the naphthalene system in indoles **49**, R = H, there is considerable variation in the CB1 receptor affinities of aminoalkylindoles **50**. The analog that is unsubstituted at C-4, JWH-195 (**50**, R = H), has modest

affinity for the CB1 receptor ($K_i = 113 \pm 28$ nM), while the 4-methylnaphthyl compound, JWH-192 (**50**, R = CH_3) has significantly greater affinity ($K_i = 41 \pm 13$ nM) and the 4-methoxy compound (**50**, R = OCH_3) has even greater affinity with $K_i = 20 \pm 2$ nM. The corresponding naphthoyl aminoalkylindoles JWH-200 (**51**, R = H, $K_i = 42 \pm 5$ nM), JWH-193 (**51**, R = CH_3, $K_i = 6 \pm 1$ nM) and JWH-198 (**51**, R = OCH_3, $K_i = 10 \pm 2$ nM) were all reported previously by the Sterling group who observed the same trend in relative CB1 receptor affinities, with the 4-methyl-1-naphthoyl- (**51**, R = CH_3) and 4-methoxy-1-naphthoylindoles (**51**, R = OCH_3) having greater affinity than the unsubstituted analog (**51**, R = H) [37].

Indoles **49**, R = H, and **50**, which are unsubstituted at the C-2 position of the indole, have significant affinity for the CB1 receptor, however 2-methylindoles, **49**, R = CH_3, have little affinity, in contrast to the 3-(1-naphthoyl)indole series in which the 2-methylindoles have only slightly reduced CB1 receptor affinity relative to the unsubstituted compounds. Molecular modeling and receptor docking studies of four indoles containing a 4-methoxynaphthyl group were carried out in order to provide a possible explanation for this considerable difference in CB1 receptor affinity in the naphthylmethane series as a function of substitution at C-2 of the indole. The compounds studied were JWH-081 (**28**, R = C_5H_{11}), its 2-methyl analog, JWH-098 (**29**, R = C_5H_{11}) and the corresponding pair of naphthylmethanes JWH-185 (**49**, R = H, R′ = OCH_3) and JWH-197 (**49**, R = CH_3, R′ = OCH_3) [57].

1-Pentyl-1*H*-indol-3-yl-(4-methoxy-1-naphthyl)methane, JWH-185 (**49**, R = H, R′ = OCH_3), $K_i = 17 \pm 3$ nM and 2-methyl-1-pentyl-1*H*-indol-3-yl-(4-methoxy-1-naphthyl)methane, JWH-197 (**49**, R = CH_3, R′ = OCH_3), $K_i = 323 \pm 48$ nM are analogs of JWH-081 (**28**, R = C_5H_{11}) and JWH-098 (**29**, R = C_5H_{11}) respectively, in which the carbonyl bridge has been replaced by a methylene group. This structural modification changes the hybridization of the bridging carbon from sp^2 in the carbonyl group, to sp^3 in the methylene group and changes the relative orientation of the naphthalene and indole rings relative to that in JWH-081, JWH-098, and WIN-55,212-2 (**4**). For JWH-185 and JWH-197, the global minimum energy conformers have the naphthalene ring oriented perpendicular to the plane of the indole nucleus [57].

1-Pentyl-1*H*-indol-3-yl-(4-methoxy-1-naphthyl)methane, JWH-185 (**49**, R = H, R′ = OCH_3), has reduced CB1 receptor affinity ($K_i = 17 \pm 3$ nM) relative to JWH-081, 1-pentyl-3-(4-methoxy-1-naphthoyl)indole (**28**, R = C_5H_{11}) with $K_i = 1.2 \pm 0.03$ nM. In the 3-(4-methoxy-1-naphthoyl)indoles the substitution of a methyl group at C-2 results in only a slight decrease in CB1 receptor affinity. JWH 081 (**28**, R = C_5H_{11}) has very high affinity with $K_i = 1.2 \pm 0.03$ nM while the 2-methyl analog, JWH 098 (**29**, R = C_5H_{11}), has only slightly lower affinity for the CB1 receptor with $K_i = 4.5 \pm 0.1$ nM. However, substitution at C-2 in the naphthylmethane series (**49**) results in a more profound 19-fold affinity loss, JWH 185 (**49**, R = H, R′ = OCH_3) has $K_i = 17 \pm 3$ nM at the CB1 receptor while JWH 197 (**49**, R = CH_3, R′ = OCH_3) has $K_i = 323 \pm 48$ nM at the CB1 receptor [57].

To gain insight into the origin of these affinity changes, modeling studies were carried out and each of these compounds was docked in a model of the active state of the CB1 receptor [57]. On the basis of CB1 receptor mutation studies in which a lysine on transmembrane helix 3 is replaced with alanine it was suggested that aromatic stacking rather than hydrogen-bonding interactions are the primary interactions for cannabimimetic indoles at CB1 [70]. It had been shown previously that this lysine is not an interaction site for WIN-55,212-2 (**4**) [39] and since JWH-081 (**28**, $R=C_5H_{11}$) and JWH-098 (**29**, $R=C_5H_{11}$), JWH-185 (**49**, $R=H$, $R'=OCH_3$), and JWH-197 (**49**, $R=CH_3$, $R'=OCH_3$) are structurally related to WIN-55,212-2 (**4**), it was hypothesized that the TMH 3-4-5-6 region of the CB1 receptor would also be the binding region for these ligands. Indoles JWH-081 (**28**, $R=C_5H_{11}$) and JWH-098 (**29**, $R=C_5H_{11}$) were docked in this region of the receptor in their lowest energy *s-trans* conformations, and indoles JWH-185 (**49**, $R=H$, $R'=OCH_3$) and JWH-197 (**49**, $R=CH_3$, $R'=OCH_3$) were docked in the same region using the global minimum energy conformer of each [57].

When the alkyl chain on nitrogen was docked in a hydrophobic-binding pocket comprised of a valine, an isoleucine, and a phenylalanine on helix 3, plus leucine and an isoleucine on helix-6, were identified which permitted simultaneous interaction of the indole and naphthalene rings with the aromatic residues in the TMH 3-4-5-6 region of the CB1 receptor active state. In this pocket, JWH-081 (**28**, $R=C_5H_{11}$) and JWH-098 (**29**, $R=C_5H_{11}$) found aromatic-stacking interactions with two tryptophan residues on helix-5 of the CB1 receptor. In this docking position, the C-2 methyl group of JWH-098 would cause no loss of affinity since this methyl group occupies an open space in the receptor-binding pocket.

The global minimum energy conformers of JWH-185 (**49**, $R=H$, $R'=OCH_3$) and JWH-197 (**49**, $R=CH_3$, $R'=OCH_3$) were docked in the same region of the CB1 receptor. However, since these compounds have conformations that orient the naphthalene and indole rings in a different manner than in 3-aroylindoles, the orientation of the ligands in the binding pocket differs from that of JWH-081 and JWH-098. Naphthylindoles **49** ($R=H$, $R'=OCH_3$) and **49** ($R=CH_3$, $R'=OCH_3$) retain the stacking interactions with the tryptophan residues on helix-5 and have an additional stacking interaction with a phenylalanine on helix-3, which involves the hydrogen at C-2 of the indole. Although the 2-methyl analog, JWH-197 (**49** ($R=CH_3$, $R'=OCH_3$) can engage in aromatic-stacking interactions with the tryptophan residues on helix-5 of the receptor, no aromatic-stacking interaction is possible with phenylalanine on helix-3 because this compound lacks the hydrogen at C-2. The nearly 20-fold drop in affinity between JWH-185 and JWH-197 is consistent with the loss of an aromatic-stacking interaction [70].

The significant affinities of indoles **49**, **50** and indenes **44**, **47,** and **48**, none of which can interact with the CB1 receptor by hydrogen bonding, strongly support the hypothesis that cannabimimetic indoles interact with the receptor primarily by aromatic-stacking interactions. The high affinity of indenes **47**

and **48**, both of which are hydrocarbons, for the CB1 receptor provides compelling evidence against hydrogen-bonding interactions playing a major role in the binding of these ligands [57]. The molecular modeling and receptor docking studies agree with this conclusion, and provide an explanation for the observation that 2-methylindole analogues JWH-196 (**49**, $R = CH_3$, $R' = H$), JWH-194 (**49**, $R = CH_3$, $R' = CH_3$), and JWH-197 (**49**, $R = CH_3$, $R' = OCH_3$) have greatly attenuated affinities for the CB2 receptor.

6.2 The CB2 Receptor

The CB2 receptor is expressed primarily in the immune system and has an overall 44% homology with the CB1 receptor [23, 24]. This homology increases to 66% in the transmembrane portion of the CB2 receptor. Although a number of detailed modeling, ligand docking, and mutation studies of the CB1 receptor have been presented, at this time relatively few similar studies of the CB2 receptor have appeared. In 1998, Tao and Abood [74] carried out a study in which a highly conserved aspartate residue in the second transmembrane domain of both the CB1 and CB2 receptors was substituted by asparginine or glutamate. When this mutation was carried out in the CB1 receptor the binding of WIN-55,212-2 (**4**) was disrupted, however, the same mutation in the CB2 receptor did not affect binding.

It has been shown that a lysine residue in the third transmembrane domain of the CB1 receptor is essential for the binding of traditional cannabinoids, but not WIN-55,212-2 [39, 69]. This lysine residue is conserved in the CB2 receptor and it was found that mutation of this lysine to an alanine did not appreciably affect the binding of several cannabinoid receptor ligands, including CP-55,940 and WIN-55,212-2 [75]. This mutant CB2 receptor was converted to a double mutant by substituting a glycine for a serine, which is also on helix 3 of the receptor. It has been suggested that this serine provides hydrogen-bonding stability in the initial mutant receptor. In the double mutant CB2 receptor [^{3}H]CP,55,940 (**2**) did not show appreciable specific binding, however specific binding of [^{3}H]WIN-55,212-2 was observed. In competition experiments using [^{3}H]WIN-55,212-2 as the radioligand, only JWH-015 (**6**), another cannabimimetic indole, competed for the binding site. Although both indoles would bind to the double mutant CB2 receptor, receptor activation was greatly reduced. Modeling and receptor docking studies were carried out using CP-55,940, none were reported using either WIN-55,212-2 or JWH-015. Another study employing mutant variations of the CB2 receptor was described by Rhee et al. in which two tryptophan residues (W158 and W172) on the fourth transmembrane domain were individually replaced with lysine, alanine, phenylalanine, or tyrosine [76]. The mutations in which either tryptophan was replaced by phenylalanine, another aromatic amino acid, did not affect binding of any of several cannabinoid receptor ligands, including WIN-55,212-2 (**4**). However, replacing

W172 with alanine completely disrupted binding to the CB2 receptor. These results are consistent with the hypothesis that aromatic-stacking interactions are important in the binding of ligands to the CB2 receptor.

In an approach to the identification of novel ligands that would be selective for the CB2 receptor, Salo et al. constructed a model of the receptor using the rhodopsin structure as a template [77]. Docking studies were carried out using four CB2 receptor ligands, including CP-55,940 (**2**) and WIN-55,212-2 (**4**). These authors concluded that hydrophobic- and aromatic-stacking interactions were more important than hydrogen bonding in their model of the CB2 receptor. In particular, the binding of WIN-55,212-2 involved a number of aromatic-stacking interactions. A similar model of the CB2 receptor was developed by Montero et al., however the only cannabimimetic indole that was employed in receptor docking studies was the inverse agonist, AM630 (**13**) [78]. These authors concluded that AM630 interacted with the CB2 receptor by a combination of hydrogen-bonding and aromatic-stacking interactions.

7 Conclusion

Modeling studies of the CB1 receptor and experiments using mutant CB1 receptors indicate that cannabimimetic indoles, and almost certainly their pyrrole and indene, analogs interact at a different site in the receptor than traditional cannabinoids and endogenous cannabinoids, such as anandamide. These studies have also shown that the cannabimimetic indoles interact with the CB1 receptor differently than traditional cannabinoids. Whereas hydrogen-bonding interactions with the receptor appear necessary for traditional cannabinoids, it is considered that aromatic-stacking interactions are important for the cannabimimetic indoles.

Although a number of workers in the field have attempted to develop a universal pharmacophore for the various classes of cannabinoid ligands, inasmuch as diverse types of cannabinoid ligands interact differently and in somewhat different regions of the CB1 receptor, it would appear to be unlikely that such a universal pharmacophore can be developed. Although the CB1 receptor and its interaction with various ligands have been studied extensively, much less has been done concerning the CB2 receptor. There is increasing evidence that this receptor has an important physiological role and the next few years should see a number of additional studies concerning the detailed structure of the CB2 receptor and the manner in which it interacts with various types of cannabinoid receptor ligands.

A great deal has been accomplished in developing the medicinal chemistry of the cannabimimetic indoles, pyrroles, and indenes in the approximately 15 years that the biological activity of these compounds has been recognized. However, much remains to be done, including a study of the SAR of the indenes and pyrroles, which should shed additional light upon the detailed interactions

of these ligands with cannabinoid receptors. Also, the development of additional ligands that is highly specific for each receptor should be carried out in order to develop further insight into the physiological role of each receptor. At present there are several highly selective indole-based compounds that are highly selective ligands for the CB2 receptor; however, there is a need for additional easily synthesized CB2 selective ligands. This will permit detailed studies of the in vivo pharmacology of these CB2 selective compounds. Such studies should be informative in terms of ultimately understanding the detailed and complex role of endogenous cannabinoids in animal physiology. Although a few indole-based CB1 selective agonists have been identified that have little affinity for the CB2 receptor, these compounds are functionally active at CB2. Highly selective ligands for the CB1 receptor with little affinity for the CB2 receptor would be of value in more fully clarifying the role of the CB1 receptor.

Although a great deal of work has been carried out concerning the SAR of the cannabimimetic indoles, further systematic studies of the effects of substituents at various position on the indole nitrogen and ring carbons, as well as on the naphthalene ring should be carried out. In addition to adding to the knowledge of the SAR at both the CB1 and CB2 receptors, these studies have the potential to lead to the development of therapeutically useful compounds.

Acknowledgments The work described in this chapter, carried out at Clemson University and included in the review, was supported by grants DA 03590 and DA15340 from the National Institute on Drug Abuse. The author thanks Drs. Billy R. Martin and Jenny L. Wiley of Virginia Commonwealth University for the pharmacological evaluation of the compounds prepared in our laboratory. The author also thanks Dr. Patricia H. Reggio of the University of North Carolina at Greensboro for the molecular modeling studies of the compounds prepared by our group. Special thanks are extended to the graduate students and postdoctorals at Clemson University who carried out the work from our group described in this chapter. These studies could not have been carried out without their contributions.

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Structure–Activity Relationships and Conformational Freedom of CB1 Receptor Antagonists and Inverse Agonists

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Abstract In the last few decades, our knowledge of cannabinoid structure–activity relationships (SARs) has increased dramatically. Computational methods have allowed us to compare active and inactive ligands for steric, electronic, and conformational similarities and dissimilarities. Knowing that there are specific G-protein-coupled cannabinoid receptors with which cannabinoids interact, we increasingly interpret changes in structure to specific alterations in ligand–receptor interaction. With additional knowledge of the CB1 and CB2 receptors' sequence, the consequences of mutations and chimeric constructs of the receptor, and the development of rhodopsin-based homology models of the cannabinoid receptors, we continue to gain insight into the interaction of cannabinoids with their receptors. Finally, our emerging appreciation for cannabinoid homo- and heterodimerization/oligomerization provides for further understanding of ligand–receptor interactions, the interaction of receptors with each other, and the interaction of G-protein-coupled receptors with their signal transduction components and other molecules affecting each receptor's dynamics. In the following review, we have attempted to characterize the emerging SARs of an important class of cannabinoid ligands: the CB1 receptor antagonists and inverse agonists. This class of compounds is showing therapeutic potential for a variety of indications, and is therefore of interest for both basic research and pharmaceutical development. Throughout this work, we have used molecular modeling, particularly quenched molecular dynamics, to illustrate particular structural modifications that have been reported for CB1 antagonists, their conformational properties, and the potential implications for receptor interaction. In this way, we hope this review serves to further guide medicinal chemists in their understanding of CB1 receptor antagonist SAR and the design of new analogs.

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_4,

Keywords Cannabinoid antagonists/inverse agonists · Structure–activity relationships (SARs) · Conformational analysis · Ligand–receptor interaction · Molecular modeling

1 Introduction

The manipulation of the endocannabinoid system for medicinal and recreational purposes has been in practice for more than 2000 years; however, only two US Food and Drug Administration (FDA)-approved cannabinoid drugs existed on the market until relatively recently: Marinol (Dronabinol) and Nabilone. Both drugs are approved for the control of chemotherapy-induced nausea and vomiting, and Marinol is also approved as an appetite stimulant for appetite loss/anorexia associated with HIV/AIDS. Although Marinol is approved for use in the United States, Nabilone does not have approval from the FDA, effectively curtailing its revenue potential. The psychoactivity of cannabinoid agonists is often cited as an adverse side effect that limits pharmaceutical interest, but some countries (including the United States) are debating or allowing/providing medicinal marijuana or orally/buccally administered cannabis solutions. For example, Sativex®, a metered and buccally administered cannabis extract produced by GW Pharmaceuticals in the United Kingdom, was approved by Health Canada in April 2005 for the relief of neuropathic pain in multiple sclerosis (MS) and was launched in Canada in June 2005 by Bayer HealthCare. In the United States, the FDA has decided to permit Phase III trials with Sativex. In the United Kingdom, the approval of this extract has been delayed; however, GW Pharmaceuticals appears determined to bring this treatment agent to the market in the United Kingdom and other European markets.

In past decades, the psychoactivity of marijuana and (–)-trans-Δ^9-tetrahydrocannabinol (THC) preparations, combined with the absence of a known mechanism of action and target receptor, clearly hindered the identification and development of additional cannabinoid ligands as therapeutics. However, more than a decade has passed since cannabinoid receptors [1, 2] and the endocannabinoid system [3–5] were discovered, and during this time period, the endocannabinoid system and CB1 receptors have been shown to comprise one of the most prevalent central nervous system (CNS) components. In addition, CB2 receptors have been found that are distributed more peripherally, such as in spleen and cells of the immune system, and cannabinoid antagonists have been discovered. CB1 receptor antagonists, which are devoid of the psychoactive effects of CB1 receptor agonists, have shown remarkable therapeutic potential and are being aggressively pursued by pharmaceutical manufacturers for a wide variety of therapeutic indications. Thus, a new millennium of cannabinoid therapeutics is in progress, where a lack of scientific evidence and prejudice is replaced by mechanism-based therapeutics and clinical trials. Indeed, the pharmaceutical industry pipelines are rapidly filling, particularly with cannabinoid receptor antagonists and inverse

agonists, and it seems only a matter of time until medicine cabinets around the world will be stocked with both prescription and over-the-counter, cannabinoid-based therapeutics.

The physiological systems modulated by the endocannabinoid system are numerous and present many opportunities for the development of much-needed therapeutic agents. For example, *in vivo* microdialysis studies with CB1 receptor antagonists/inverse agonists in the medial prefrontal cortex of rats have shown that these agents enhance the release of catecholamines, particularly dopamine, and also exhibit procholinergic properties, making them candidates for the treatment of psychosis, affective, and cognitive disorders. In agreement with this hypothesis, cannabinoid antagonists/inverse agonists have attenuated phenylcyclidine- and d-amphetamine-induced hyperlocomotion, without affecting locomotor activity when administered alone, and have decreased immobility in the forced swimming test [6]. However, the failure of these same compounds to reverse disruptions in paired-pulse inhibition, hyperactivity, or stereotypy induced by noncannabinoid psychotomimetic agents suggests that blockade of the CB1 receptor is not always sufficient for antipsychotic activity [7]. Interestingly, a significant (64%) increase in radioligand binding to the CB1 receptor was found in postmortem tissue from schizophrenics as compared to control, supporting a role for the endogenous cannabinoid system in the anterior cingulate cortex in the pathology of schizophrenia, particularly in relation to negative symptoms [8]. Similarly, the high concentration of CB1 receptors and the presence of endocannabinoids in the hippocampus imply a role for the endocannabinoid system in learning and memory processes. As a result, Alzheimer's disease has also been viewed as a potential therapeutic indication for cannabinoid modulation. This is a reasonable target when one considers that cannabinoid agonists disrupt learning in rats [9] and squirrel monkeys [10] responding under a repeated acquisition procedure, and that cannabinoid antagonists block this disruption. However, cannabinoid antagonists/inverse agonists, *when administered alone to animals*, have been reported to impair [10], to have no effect on [9, 11], or to improve cognition [12, 13]. Factors of the experimental design, including species, dose, or temporal variables that affect acquisition, consolidation, recall, and extinction, are often cited to explain these discrepancies. Studies that show some memory improvement have often utilized a novel delay version of the radial-arm maze task [14]. The large number of receptors in the substantia nigra has also led several research groups to examine the effects of cannabinoid agonists and antagonists in movement disorders, such as Parkinson's disease and Huntington's chorea. In one study, SR141716 (Rimonabant, or Acomplia, **1**) demonstrated efficacy in a rodent model of Parkinson's disease [15], but only when severe nigral lesioning was present (>95% cell loss); however, in primates with 1-methyl-4-phenyl-1,2,3,6-tetrahydopyridine (MPTP)-induced lesions, SR141716 was unable to alleviate the motor deficits of Parkinsonism [16]. Finally, cannabinoid antagonists/inverse agonists have been reported to show particular promise as a smoking/drug abuse cessation agent [17–26] and as

antiobesity/metabolic syndrome treatment agents [27–29]. The first potent cannabinoid antagonist to be reported [30], SR141716, has recently received approval for the treatment of obese patients or overweight patients with associated risk factors, such as type 2 diabetes or dyslipidemia from the Committee for Medicinal Products for Human Use of the European Medicines Agency (EMEA). Intriguingly, the observed improvements in HbA1c, HDL cholesterol, and triglycerides were beyond that expected from weight loss alone. The antiobesity activity of cannabinoid antagonists/inverse agonists has also been claimed in combination therapy approaches with lipase inhibitors [31], $ppar_{\alpha}$ agonists [32], opioid antagonists [33], and other molecules for controlling appetites. Although other therapeutic indications continue to be described for cannabinoid receptor antagonists and inverse agonists, the marked effect of these molecules on the CNS, metabolic parameters, and weight loss has certainly gathered interest from a wide variety of pharmaceutical manufacturers. Regardless of the particular indication, the structure–activity relationships (SARs) of known CB1 receptor antagonists are rapidly expanding with SR141716-like pyrazole analogs, as well as with an increasing number of novel chemotypes, which will be further described and characterized below.

As stated previously, our goal in this work is to review the structural diversity of CB1 receptor antagonists/inverse agonists and to present the reader with a perspective of the conformational preferences shown by particular analogs of interest. We have used quenched molecular modeling previously to derive conformational information for comparative molecular field analyses, and ultimately, to derive quantitative SARs for cannabinoid antagonists. This approach involves molecular-dynamics simulations on each energy-minimized analog at 2000 K. During the molecular dynamics simulation, the molecule was heated from 0 to 2000 K at 100 K steps lasting 10 ps each, with snapshot conformations taken at 10-ps intervals. Upon reaching 2000 K, the molecule was held at this temperature for 1000 ps, while additional snapshots were acquired at 10-ps intervals. Each of the snapshot conformations obtained for a particular analog was energy minimized again using a conjugate gradient of 0.01 kcal/mol or a maximum of 100,000 iterations as termination criteria, yielding a group of 139 energy-minimized conformers per compound. After these simulations had been performed, all conformations from each of the analogs were overlaid using a single template molecule. Because the pyrazole ring system of SR141716 had a reasonably close corresponding central ring system in most of the analogs, the pyrazole ring atoms were used for atom-by-atom root mean square distance minimization. This alignment positioned all of the molecules in the same three-dimensional space and superposed the central ring systems to as great an extent as possible. The conformations were quite diverse for certain molecules, while other molecules repeatedly yielded a small number of similar conformations. To visualize the differences in conformational mobility, figures are presented that overlay the conformations within 10 Kcal/mol from the global energy minimum conformation of a particular molecule to the pyrazole ring system of SR141716. Finally, the figures are presented in orthographic

visualizations, where the conformational overlays are presented in two views, with the image on the left presenting a view of the conformers, and the image on the right presenting a view of the same overlay rotated 90° to the right. The atoms are colored in the traditional manner, with carbon atoms colored white, oxygen atoms colored red, and nitrogen atoms colored blue, etc. It is in this manner that we have attempted to provide the reviewer with information regarding both the SARs and conformational mobility of CB1 cannabinoid receptor antagonists and inverse agonists.

2 Structural Diversity of Cannabinoid Agonists/Inverse Agonists

2.1 Conformationally Constrained Analogs of SR141716

With SR141716's structure (Fig. 1**a**), the aryl rings at positions 1 and 5 are relatively free to rotate with coordinated, or cogged, motion. There is also a small energy barrier to rotation of the monochlorinated ring system due to the methyl substituent at the 4 position. Due to these steric interactions, as well as

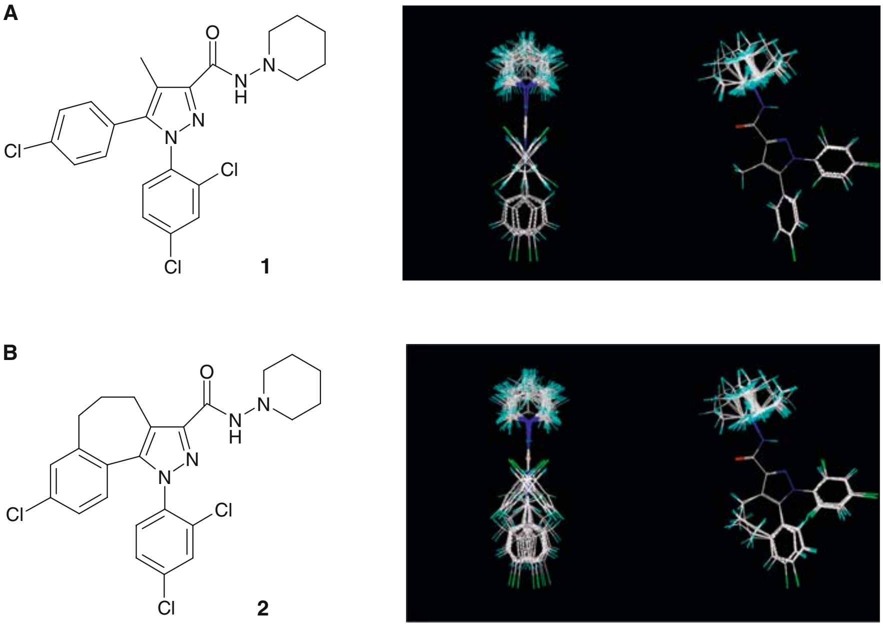

Fig. 1 **(a)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of SR141716 (**1**). **(b)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of NESS 0327 (**2**). ([37], and also reported in [36]). **(c)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of O 1248 (**3**) [39]. **(d)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of compound **4** [40].

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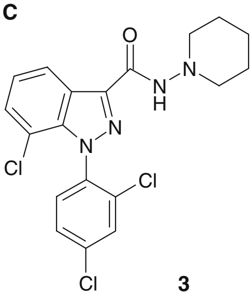

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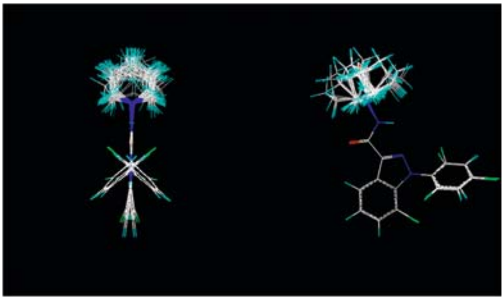

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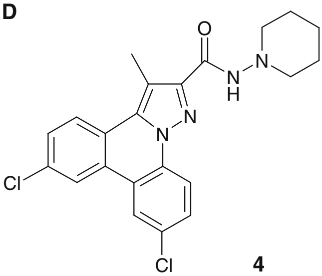

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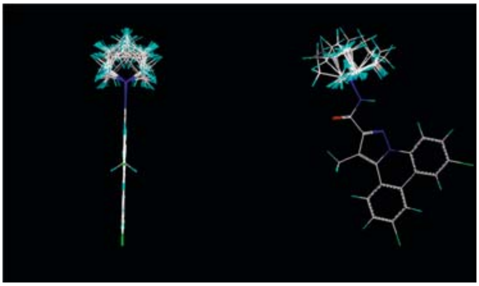

Fig. 1 (Continued)

electrostatic factors, each aromatic ring prefers local energy minima conformations at 4 torsion angles. The dichlorinated ring system prefers torsion angles of 40/–40 and 140/–140 with respect to the pyrazole ring, while the monochlorinated ring system prefers torsion angles at 60/–60 and 120/–120. Again, each angle is readily accessible at room temperature with cogged rotation of the aryl rings. The rotational freedom of these aromatic rings has been a target for modification by several investigators. For example, constraint of the monochlorinated ring through a carbon bridge from an *ortho* carbon of the ring to the 4-position of the pyrazole has been reported to produce some unusual pharmacological properties [34–36]. The Murineddu laboratory found that when only one carbon serves as the bridge, with the monochlorinated ring system pulled into the plane of the pyrazole, and the dichlorinated ring system relatively free to rotate, the CB1 affinity is greatly reduced (~2000 nM in a murine CB1 preparation, mCB1) and the CB2 affinity is enhanced dramatically (~0.3 nM at mCB1). When two carbons are employed in the bridge, the monochlorinated ring system can deviate from planarity slightly (~20°) and the dichlorinated ring system can still rotate relatively freely. In this instance, the mCB1 affinity is only modestly reduced (~12 nM), and there is little change in the mCB2 affinity (~200 nM), as compared to SR141716. However, when three carbons are employed and the monochlorinated ring can deviate further (~30°) from planarity with the pyrazole, the Murineddu group found the mCB1 affinity

increased to an astonishing 350 fM, while the mCB2 affinity was moderately increased to ~20 nM [34, 35, 37]. Interestingly, this compound (NESS0327, **2**, Fig. 1**b**) had been synthesized and characterized by Stoit and colleagues several years earlier and found to have nM affinity (126 nM), as opposed to fM affinity, and to be approximately equipotent to SR141716 at blocking CP55940-induced changes in cAMP in Chinese hamster ovary cells expressing the human CB1 receptor [36]. We too have synthesized and tested this compound in our laboratory and found it to possess nM affinity in CB1 transfected cell lines, as well as in mouse brain membrane preparations ($K_i \sim 100$ nM, unpublished observation). The large discrepancy between these affinities is compounded by the fact that the Murineddu laboratory reported several additional analogs with relatively modest structural changes from NESS0327 that also were characterized with low pM affinity. The SARs reported within these NESS0327 analogs were surprisingly different from those known for SR141716 as well. For example, SR141716 has ~1 nM affinity at the hCB1 receptor, and removal of the chlorine atom on the monochlorinated ring decreased the affinity to ~100 nM. However, when the same chlorine atom is removed from NESS0327, the decrease in affinity to the reported value of ~168 nM is almost 500,000-fold. The Stoit group had also made this molecule earlier and reported a reasonably similar K_i of ~400 nM and approximately equal potency to SR141716 in a functional cell assay (blockade of CP55940-induced cAMP formation) in hCB1-transfected CHO cells [36]. Thus, although these ring-constrained molecules as reported by Murineddu are the highest-affinity compounds reported to date, there are inconsistencies between laboratories in NESS0327 affinity values that require further clarification. Finally, we noted that if four carbons were used in the bridge, the energy barrier to rotation of the monochlorinated ring across the plane of the pyrazole was increased such that atropisomers were formed at room temperature. These mirror image conformations were apparent on the NMR timescale, as different chemical shifts were observed for geminal protons on the bridge carbons. The K_i of the racemic mixture was of ~11 nM in competition assays with [^{3}H]SR141716, while the K_i calculated for this compound in competition assays with [^{3}H]CP55940 in hCB2 was more than 400 nM [38]. Efforts to synthesize, separate, and test these and other atrope isomers may provide greater detail regarding ligand recognition within cannabinoid receptors.

An alternative approach to lock the monochlorinated ring system in a particular position involved fusing the central pyrazole group of SR141716 with its 5-(4-chlorophenyl) substituent to form a central indazole ring, O-1248 (**3**, Fig. 1**c**) [39]. Its relatively low affinity (~500 nM) cannot be solely attributed to constraining the ring in the plane of the pyrazole, because this molecule does not constrain the overall geometry or conformation of the monochloro ring to a position that closely approximates that occupied by the same ring in SR141716. One can see this structural difference by comparing the location of the chlorine atom in the various low-energy conformations of SR141716 (Fig. 1**a**) with the location of the chlorine atom in O-1248.

Constraint of both ring systems has been reported to occur following photochemical reaction of SR141716. Upon irradiation with a 450 W Hanovia mercury arc lamp, this CB1 cannabinoid antagonist undergoes a photocyclization reaction to yield a pyrazolo[1,5-f]phenanthridine ring system, giving rise to compound **4** [40]. This compound (Fig. 1**d**) has a structure wherein the aryl ring systems are locked into the plane of the pyrazole ring, yet it retains reasonably high affinity for the CB1 receptor (CB1 K_i = 48.0 nM) and is still selective for the CB1 receptor (CB2 K_i = 3340 nM). However, it has no effect on GTP-γ-[^{35}S] binding when tested alone; acting instead as a silent antagonist in this particular assay.

At the 3-position, SR141716 energetically favors an *s*-trans configuration of the carbonyl oxygen and the pyrazole ring nitrogen (or dichlorophenyl ring), with the aminopiperidine substituent free to assume several low-energy conformations. Synthesis and testing of several analogs of SR141716, combined with molecular modeling and receptor docking studies, suggests that this *s*-trans conformation of the carbonyl oxygen with the pyrazole ring nitrogen allows for a critical hydrogen-bond interaction between the oxygen atom in SR141716 and lysine K3.28(192) in the CB1 receptor [41]. However, conformational constraint and structural modifications of this region have recently been reported where molecules were synthesized that constrained the carboxyamide oxygen atom in the *s*-cis position and still retained reasonable affinities. For example, the constrained compound **5** reported by Carpino et al. (Fig. 2**a**), which has only hydrogen-bond accepting capability, exhibited surprisingly high affinity (20 nM) despite the *s*-cis conformation and the poor overlap of the cyclohexyl group with the piperidinyl group in the low-energy conformer of SR141716. This result suggests that this analog may bind differently than SR141716 in hCB1, with the cyclohexyl group occupying a separate hydrophobic pocket, distinct from that for the 1-piperidinyl group in SR141716, and/or the

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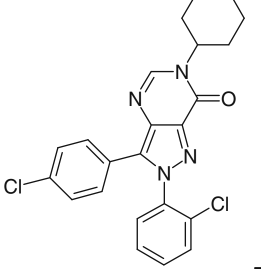

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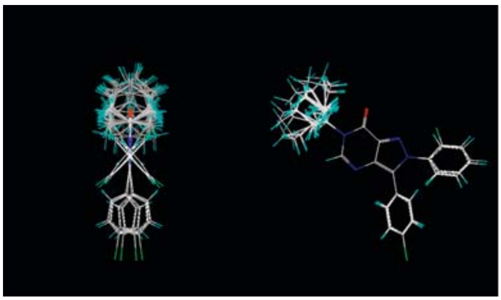

Fig. 2A **(a)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of compound **5** [42]. **(b)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of the pyrazole **6** [47]. **(c)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of SR144528 (**7**). **(d)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of triazole **8** [49]

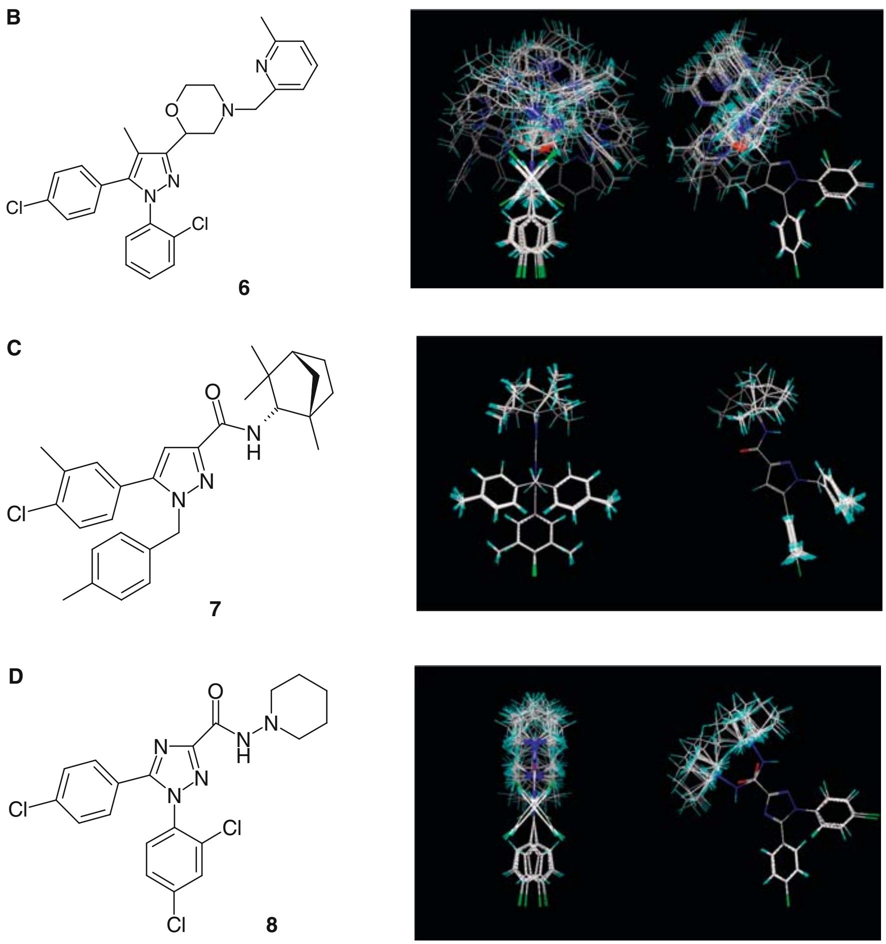

Fig. 2 (Continued)

hydrogen-bond accepting groups forming different networks of hydrogen-bond interactions. One must also consider that the conformationally constrained molecules may induce conformational changes in the receptor to accommodate the piperidinyl rings in the same hydrophobic pocket, as well as to allow alternative hydrogen bond formation.

2.2 Alterations of Aryl/Pyrazole Substituents

Follow-on compounds of Sanofi–Aventis include the structural analog SR147778, with an ethyl instead of a methyl at the 4 position, and a bromine for chlorine substitution on the aryl ring at the 5-position. These

modifications are consistent with the SARs for high-affinity binding at the CB1 receptor, which favors a *para*-substituted phenyl ring at the pyrazole 5-position and a 2-chloro- or 2,4-dichloro-phenyl substitution pattern at the pyrazole 1-position [43]. Removal of the *para*-substituted halogen to the phenyl causes more than a 10-fold decrease in affinity. Replacement of the chlorine with bromine or iodine has little affect on affinity, whereas substitution of the chlorine with polar NO_2 or NH_2 groups, or simply moving the *para*-substitutent to the *ortho* position, also tends to cause marked decreases in affinity [44]. More dramatic modification of the pyrazole substituents of SR141716 has led to some surprising changes in affinity, efficacy, and/or receptor selectivity. For example, Wiley et al. retained the central pyrazole structure of SR141716 and replaced the 1-, 3-, 4-, and/or 5-substituents with alkyl side chains or other substituents known to impart potent *agonist* activity in tricyclic cannabinoids [45]. In pyrazole analogs in which the carboxyamide group of the 3-substituent of SR141716 was replaced with an alkylether linker coupled to terminal cyclic systems, affinity was greatly decreased when the terminal piperidine group of SR141716 was retained. Interestingly, when the terminal ring systems were naphthalene (O-852), 4-fluorophenyl (O-889), and 2,4-difluorophenyl (O-1043), compounds with moderate affinity ($\sim$50 nM) were obtained. When the carboxyamide group was retained or replaced with a ketone at the 3-position, but the terminal piperidine ring system was replaced with alkyl side chains, compounds with moderate affinity at the CB1 receptor were again observed. The highest affinity was observed with the pentyl, and shortening or extension of the alkyl chain rapidly decreased affinity. This finding is consistent with other studies that found that branching of the carboxyamide side chain at the 3-position could increase affinity [46]. However, the pentyl analog and several other alkyl analogs displayed partial agonist activity in the mouse tetrad assessment (albeit none of the analogs produced the full spectrum of *in vivo* effects seen with full agonists) [45]. Furthermore, none of the 3-substituted analogs produced antagonist effects when tested in combination with 3 mg/kg Δ^9-tetrahydrocannabinol (Δ^9-THC). In contrast, antagonism of Δ^9-THC's effects was observed with substitutions at positions 1, 4, and 5, and no agonist or partial agonist effects were observed in these series. These results suggest that the structural properties of 1- and 5-substituents are primarily responsible for the antagonist activity of SR141716 [45]. An additional observation made as the carboxyamide alkyl side chain is extended beyond seven carbons is that the compounds appear relatively less able to displace [^{3}H]WIN55212-2 from the CB1 receptor as compared to their ability to displace [^{3}H]CP55940 or [^{3}H]SR141716 [46].

Variations at the 3-position of the pyrazole ring have also been the subject of patents filed by Dow and Hammond at Pfizer [47]. They described pyrazole analogs where the carboxamide was replaced by other polar groups, such as an amino alcohol, ketone, morpholino ring, or an imidazole (**6**, Fig. 2**b**). The authors proposed that these substituents serve as hydrogen-bonding acceptors, as does the carbonyl oxygen in SR141716. This analog and others possess

relatively large substituents at this position, yet retain reasonable affinity for the hCB1 (K_1 of **6** ~80 nM), suggesting that the receptor recognition site is able to accommodate additional steric bulk in this region. Ultimately, substitution of each substituent of the pyrazole can lead to CB2 selective molecules, such as SR144528 (**7**, Fig. 2**c**), which was also reported by Sanofi [48].

2.3 Triazoles

Compounds with a triazole core have been reported by at least three different groups [49–52]. Although these triazoles, in general, have lower affinities for the CB1 receptor (K_i = ~400 nM) than the corresponding pyrazoles, they provide valuable information on the SARs of CB1 receptor antagonists. Initially, Dyck et al. suggested that the reduced affinity of these triazoles (e.g., **8,** Fig. 2**d**) was due to the lack of the methyl group, as present on the pyrazole core in SR141716, which either fits in a small binding pocket or impacts the preferred orientation of the 4-chlorophenyl ring and/or the carboxamide [49]. Indeed, their molecular modeling and superimposition of the triazole core of **8** with the pyrazole core of SR141716 indicated that the low-energy conformation of the carboxamide in the triazole is not the low-energy conformation observed in the corresponding pyrazole. Specifically, an *s*-cis conformation was observed in the triazole (N2 = C3–C = O), in which the carboxamide oxygen is on the same side as the triazole's N2 and the dichlorophenyl group. In our molecular modeling studies on **8**, both *s*-cis and *s*-trans were observed to be within 10 kcal/mol of the global energy minimum *s*-cis conformation (see Fig. 1**c**), while only s-trans configurations were observed within 10 kcal/mol of the global energy minimum with SR141716 (Fig. 1**a**). The other ring systems are quite comparable. Jagerovic and co-workers suggest that this different spatial orientation of the carboxamide contributes to the low affinity of the triazoles [50]. In a different approach, Lange and colleagues from Solvay Pharmaceuticals used a receptor-based alignment and found that trizole analogs could overlay quite readily with SR141716, as well as noted that there was no significant preference for the *s*-trans or *s*-cis conformers, since both can form hydrogen bonds between the triazole nitrogens and the NH in the carboxamide because the molecule is *symmetrical* with respect to the nitrogens (N2 and N4) [51].

The triazole **9** (Fig. 3**a**), in which the carboxamide is replaced by a hexyl group, was also evaluated by the Jagerovic team [52]. Here again, we found that the low-energy conformations of the aryl ring systems of SR141716 are closely mimicked by compound **9**. However, this compound showed moderate affinity (K_i = 0.8 μM) for CB1 receptors and silent antagonist activity both *in vivo* and in functional assays, such as mouse vas deferens and guinea pig ileum preparations [52]. The authors claimed that the compound agreed with certain pharmacophore elements commonly described for cannabinoid ligands, but that the absence of the carbonyl in **9** precludes hydrogen-bond formation

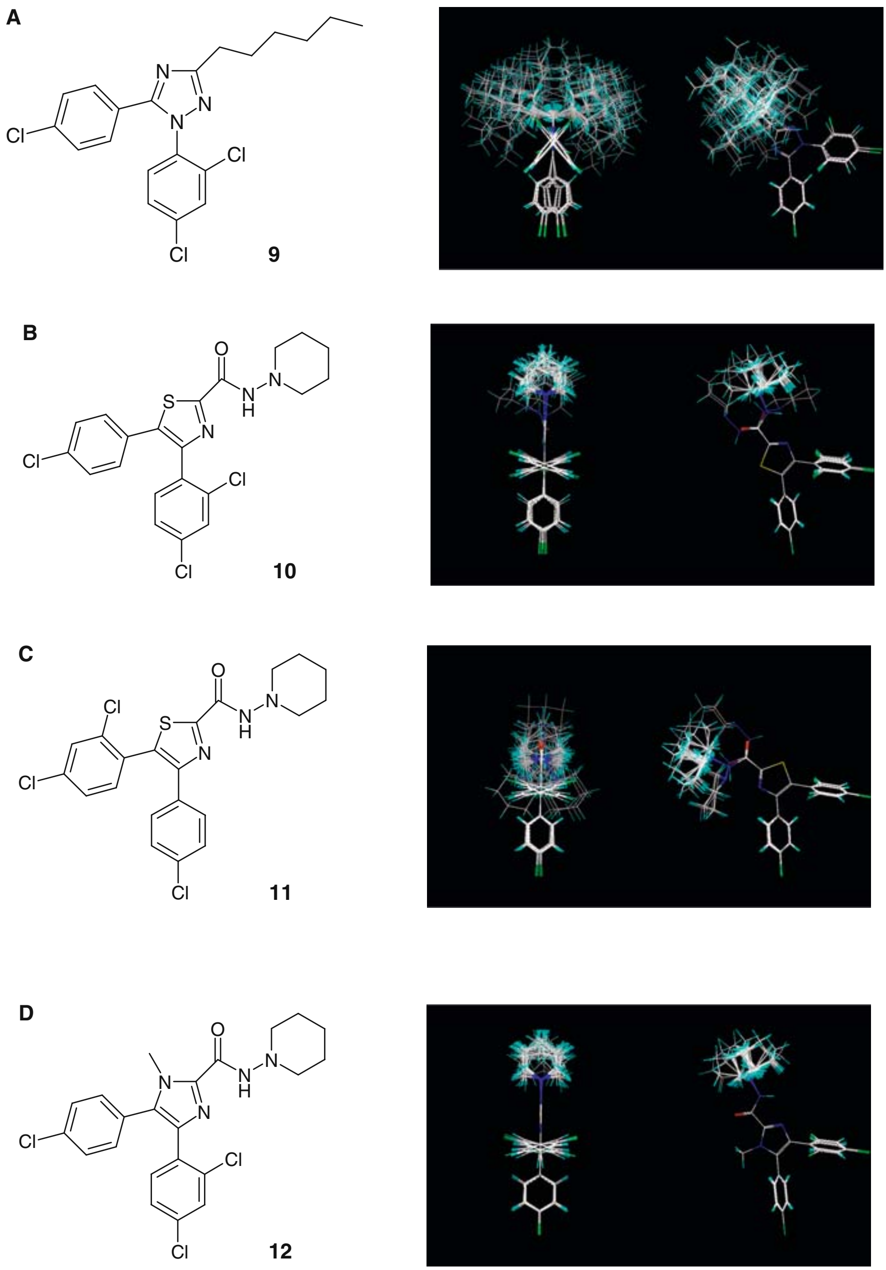

Fig. 3 **(a)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of triazole **9** [51]. **(b)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of thiazole **10** [52]. **(c)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of thiazole **11** [52]. **(d)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of imidazole **12** [53]

with the CB1 receptor, as described for SR141716 [41], thus decreasing its binding affinity.

2.4 Thiazole and Oxazoles

Thiazole and oxazole bioisosteres have been reported by several research groups. Similarly to the triazoles, these compounds show less potency than the corresponding 4-methylpyrazoles. For example, a research group at Merck obtained IC_{50} of 260 nM for an oxazole with the same phenyl substitution pattern (parachlorophenyl and 2,4-dichlorophenyl) as SR141716. Another interesting pair of thiazole compounds with similar substituents as SR141716 was evaluated: compound **10** shown in Fig. 3**b** (K_i = 227 nM) was found to have significantly higher affinity than compound **11** shown in Fig. 3**c** (K_i > 1000 nM) [52]. This difference was explained by the receptor-based alignment. The H-bond formed between the thiazole N with the NH in amide makes the *trans* conformation more favorable in **10**, that is, the dichlorophenyl ring on the opposite side of the carboxamide oxygen, despite the larger electron repulsion between the sulfur atom and carbonyl oxygen. In **11**, however, such an H-bond makes the *cis* conformation more stable. In order for optimal receptor interaction as proposed for SR141716, **11** has to adopt a less-stable *trans* conformation when binding to the receptor. Such a transformation requires additional energy, thus decreasing the binding affinity of this compound. One might also note that the low-energy conformations of the 2,4-dichlorophenyl ring system and the parachlorphenyl ring system appear to be different than those observed with SR141716 (note the larger torsion angles of the dichloro ring system in Figs. 3**b, c** as compared to the same torsion angles in SR141716 in Fig. 1**a**).

2.5 Imidazoles

The imidazoles bioisosteres represent a series of SR141716 analogs that can retain high affinity for the CB1 receptor. One SAR study of 4,5-diaryl imidazoles was performed by Plummer from Merck [53]. The optimal compound, **12** (Fig. 3**d**), is almost structurally identical to Rimonabant and displays similar affinity (6.1 nM). Similar to what is seen with pyrazoles, the removal of the N-methyl or the chlorine from the dichlorophenyl decreases the potency of these compounds. The replacement of piperidine with a cyclohexyl group increases the affinity modestly.

The Dyck team and Plummer team independently synthesized and studied the SAR of the 1,2-diarylimidazoles [49, 53]. Again, the importance of the methyl group on the imidazoles was demonstrated by both groups. Based on the similar binding affinity of the 1-methylimidazoles and SR141716, Dyck

proposed that the binding of these compounds to the CB1 receptor relies more on the substituents than the nature of the hetero core itself [49].

2.6 Pyrroles

1,5-Diarylpyrroles, patented by several groups, cause significant decreases in food intake after oral administration [54, 55], as does SR141716. One interesting example is **13** (Fig. 4**a**), in which a bridge between the amide nitrogen and the pyrrole 2-position was constructed. Although no specific binding data is

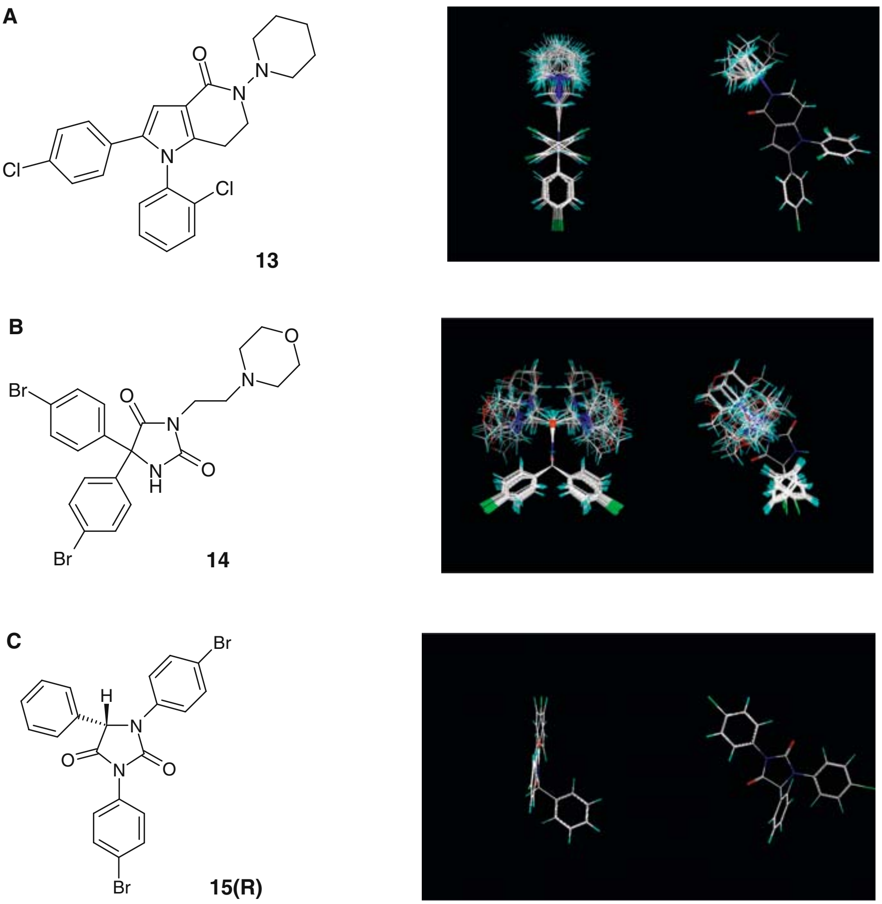

Fig. 4 **(a)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of the 1,5-diarylpyrrole **13** [54]. **(b)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of DML20 (**14**) [57]. **(c)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of **15R** [60]. **(d)** Chemical structure (left) and orthographic views of the low-energy conformations (right) of **15S** [60]

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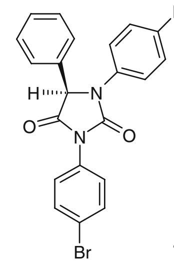

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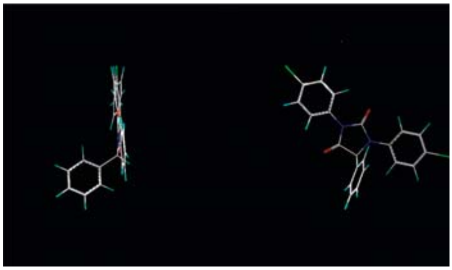

Fig. 4 (Continued)

available, **13** might be expected to be active due to the locked s-*trans* conformation (carbonyl oxygen and 2-chlorophenyl).

2.7 Hydantoins

3-Alkyl-5,5′-diphenylimidazolidinediones have been shown to be antagonists/inverse agonists at the CB1 receptor of moderate affinity [56]. DML20 [57] (**14**, Fig. 4**b**) had the highest affinity in the series with a K_i of ~70.3 nM and acted as neutral antagonists in rat brain membrane preparations and as inverse agonists on human CB1 cannabinoid receptors expressed in CHO cells [58]. The same group recently synthesized and evaluated the 5,5′-diphenyl-2-thioxoimidazolidine-4-ones [59]. The bioisosteric replacement of the oxygen with sulfur increases the affinity without affecting their functional characteristics. An increase of affinity was also observed with the introduction of para substituents on the 5-phenyl rings and with lengthening of the alkyl group at the 3-position. 1,3,5-Triphenylimidazoline-2,4-diones and 1,3,5-triphenyl-2-thioxoimidazolidin-4-ones were also recently described [60]. An interesting structural feature of these compounds, which has been confirmed by X-ray diffraction, is that the H's at the ortho positions of the 1,3 aromatic rings form short intramolecular bonds with the 2-carbonyl oxygen. These intramolecular H-bonding interactions influence the conformation of the molecules and could potentially affect the binding to the receptor. Binding studies of these compounds, using CHO cells expressing hCB1 or hCB2, show that substitution of the 3-position with an aromatic ring is required. Indeed, aromaticity of the 1,3-substituents appears very important, because much lower displacement (25%) was observed when the two groups are cyclohexyls, compared to 57% for phenyls. Moreover, substitution with Cl or Br at the para position, as in **15** (311 nM at rCB1 (cerebellum) and 243 at hCB1 transfects), largely increases the affinity. Substituents on the para position of C5 phenyls, however, results in a decrease in affinity. The corresponding 1,3,5-triphenyl-2-thioxoimidazolidine-4-one compounds were prepared and studied in parallel. Opposed to the 3-alkyl-5,5′-diphenyl derivatives, the substitution of the carbonyl with a thiocarbonyl resulted in roughly a sevenfold decrease in affinity.

Lambert discovered, based on a receptor-based alignment, that the interaction pattern of the two enantiomers of **15** is governed by the stacking of the two bromophenyls to the receptor aromatic domains, especially with F3.36, Y5.39, and W5.43 (see Figs. 4**c**, 4**d**). The S-enantiomer positions the 4-carbonyl to form an H bond with K3.28, which has been demonstrated to be responsible for the higher affinity in SR141716. In the R isomer, however, in order for the aromatic stacking interaction, the carbonyl group at the 4-position points away from K3.28. Therefore hydrogen bonding is not possible, and a lower-binding affinity is resulted for the R isomer. The authors claimed that, although no supporting binding data for each isomer individually was provided, the binding difference reflected the importance of the stereochemistry of the receptor. It is also noticed that the lipophilic pocket formed by F2.67, F2.64, H2.65, and F2.61, which is occupied by the piperidine ring in the case of SR141716, is left unfilled. They suggested that this might be responsible for the lower affinity for these compounds.

2.8 Pyrazolines

Lange reported a series of 3,4-diarylpyrazolines as cannabinoid receptor ligands. As a chiral center is present at the 4-position on the pyrazoline, racemates were obtained, which were subsequently separated and tested [61]. SLV319 (**16**) displayed the highest affinity (7.8 nM), while the epimer shows much lower activity (894 nM). This compound also demonstrated sevenfold higher CB1/CB2 selectivity (~1000) than Rimonabant. Conformational analysis by molecular modeling revealed the flexibility of this molecule, with most of the population lying within 4 kcal/mol compared to the minimum energy conformation (Fig. 5**a**). Receptor-based modeling shows the presence of a hydrogen bond between one of the SO_2 oxygen and the D6.85(366)-K3.28(192) salt bridge. An additional hydrogen bond of the other oxygen

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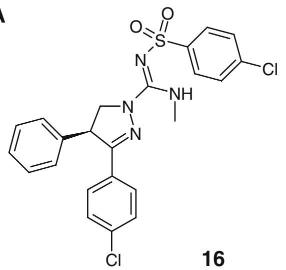

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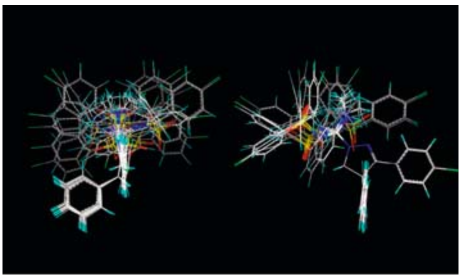

Fig. 5 (a) Chemical structure (left) and orthographic views of the low energy conformations (right) of the 3,4-diarylpyrazoline SLV319 (**16**) [61]. **(b)** Chemical structure (left) and orthographic views of the low energy conformations (right) of O-1803 (**17**) [39]. **(c)** Chemical structure (left) and orthographic views of the low energy conformations (right) of **18** [63]. **(d)** Chemical structure (left) and orthographic views of the low energy conformations (right) of **19** [62].

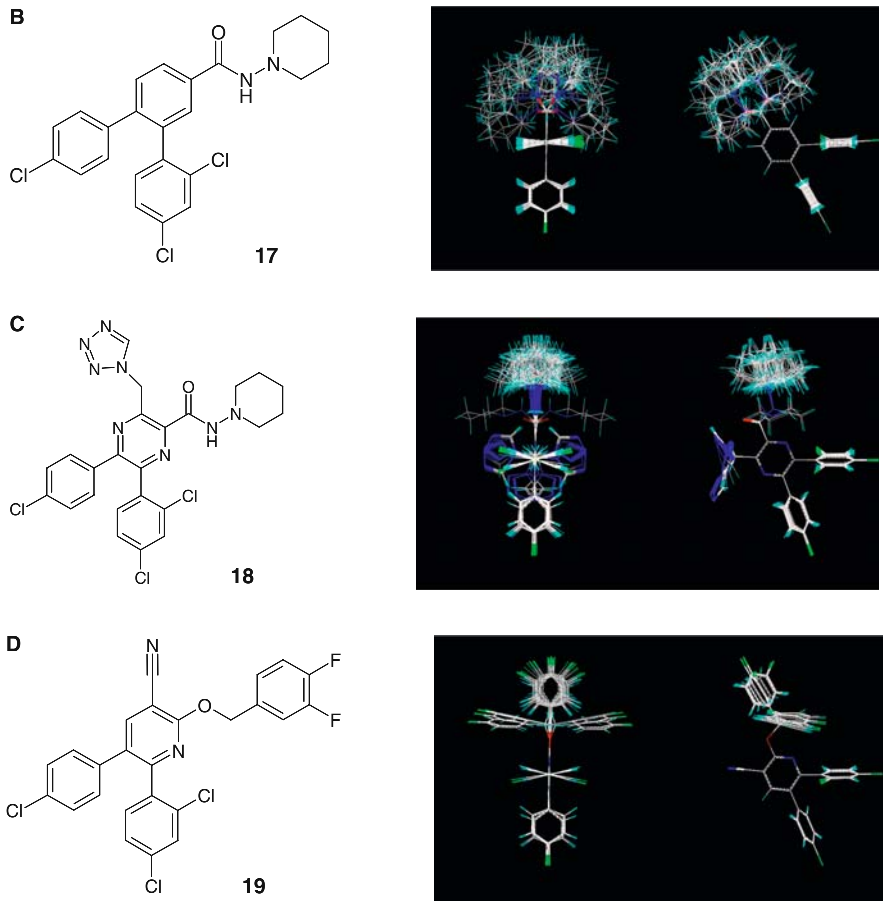

Fig. 5 (Continued)

atom with Ser383 further enhanced the binding of SLV 319 to the receptor. This observation is supported by the fact that the replacement of SO_2 with a carbonyl group reduced the affinity by threefold. The two aromatic rings attached to the pyrazoline core fit into two pockets formed by lipophilic residues. Although SLV319 has the appropriate alignment, its enantiomer has the phenyl ring pointing away from the lipophilic pocket, therefore attenuating the stacking interactions.

2.9 Six-Membered Ring Systems

Analogs in which the pyrazole core is replaced with six-membered rings have emerged over the past few years. Martin and collaborators have

substituted the pyrazole with a simple phenyl, producing O-1803 (**17**), as shown in Fig. 5**b** [39]. A significant decrease in affinity ($K_1 \sim 113$ nM) was observed, which led the authors to suggest that a pyrazole or similar nitrogen-containing back bone is involved in ligand–receptor interaction. It is also interesting to note the difference in the conformation of the aryl ring systems from SR141716.

2,3-diarylpyrazines have also been disclosed in patents, and in this series, the addition of the heterocycle nitrogens and more polar substituents, such as the tetrazol-1-ylmethyl in compound **18** (Fig. 5**c**), produced compounds of high affinity and potency. Indeed, **18** possessed an IC_{50} below 2 nM in an hCB1 receptor binding assay using CP55940.

Several patent applications have been filed on 2,3-diarylpyridines and pharmacological results subsequently published by Merck. Quite a few compounds in the series appear promising. Compound **19** (Fig. 5**d**), displayed high CB1 receptor binding affinity (IC_{50} hCB1 = 1.3 nM) and high selectivity for CB1 over CB2 receptors (400-fold) [62]. A moderate oral absorption (F = 27%) was also observed, accompanied by slow brain penetration and a low brain-to-plasma ratio. Food intake reduction and weight loss were apparently, and perhaps surprisingly, not significantly affected. In the SAR studies, the Merck researchers demonstrated that the same chlorination pattern on the two aromatic rings as SR141716 is critical for optimal binding. The benzyloxy presumably occupies the same area in the receptor as the piperidine through a lipophilic interaction without any direct aromatic requirements. The polar cyano group can be replaced with an amide without loss of affinity, particularly when it is a secondary amide. The excellent binding affinity suggests that the corresponding carboxamide in SR141716 can be divided into a polar group and a lipophilic functionality. The moderate hCB1 inverse agonism was attributed to the absence of the neighboring methyl group; the difference in three-dimensional orientation of the substituents on the six-membered ring, as compared to five-membered pyrazole in SR141716; and/or electronic difference between the pyridine nitrogen and pyrazole nitrogen.

2.10 Azetidines

Initially developed as a treatment agent for anxiety and epilepsy, the azetidine-1-carboxamides displayed high binding affinity for CB1 receptors and unexpected efficacy as antiobesity agents [64]. In the competition assay with [^{3}H]SR141716 Compound **20**, enriched in one enantiomer, shows high affinity at the cannabinoid receptor (K_i (hCB1) value of 0.6 nM). In the overlay of its low-energy conformations shown in Fig. 6**a**, one can see that the aromatic rings occupy similar locations as those occupied by the aromatic rings in SR141716 (compare Fig. 1**a**), and that the remainder of the molecule is free to traverse a similar region as that occupied by the aminopiperidine in SR141716.

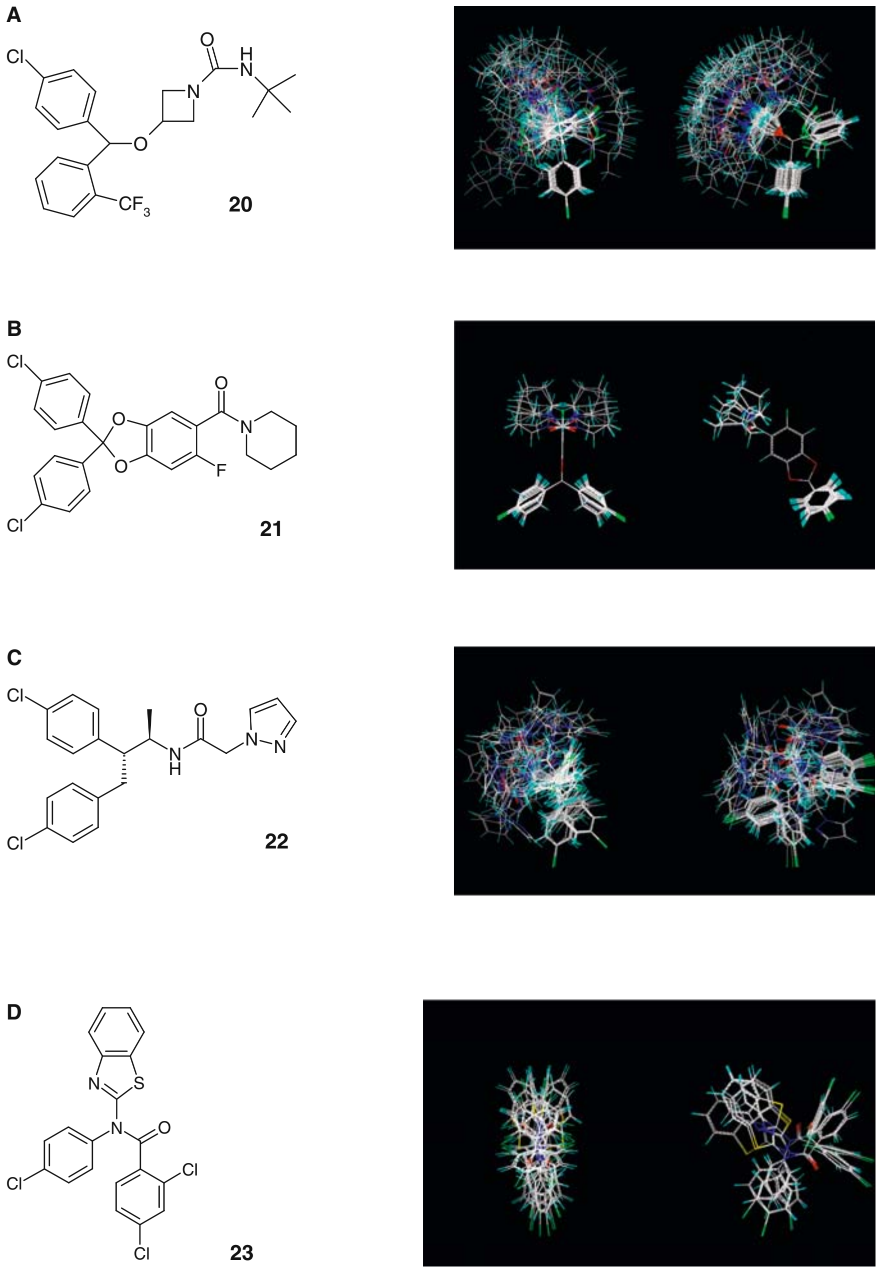

Fig. 6 **(a)** Chemical structure (left) and orthographic views of the low energy conformations (right) of **20** [64]. **(b)** Chemical structure (left) and orthographic views of the low energy conformations (right) of **21** [65]. **(c)** Chemical structure (left) and orthographic views of the low energy conformations (right) of **22** [66]. **(d)** Chemical structure (left) and orthographic views of the low energy conformations (right) of **23** [67].

2.11 Benzodioxole

A patent on 1,1′-diarylbenzodioxoles such as compound **21** has been published [65]. These compounds were claimed to be CB1 receptor antagonists. They are claimed to display binding affinity to the CB1 receptor less than 2 μM and selectivity more than 10-fold against the CB2 receptor. In the alignment presented in Fig. 6**b**, one can see that the piperidine ring and carbonyl oxygen may provide elements for receptor recognition similar to those in the carboxyamino–piperidine system of SR141716, while the aryl rings in this molecule prefer conformations similar to those in the hydantoin DML20 (Fig. 4**b**).

2.12 Substituted Amides

A series of compounds with open chain structure were also claimed in a patent application in 2003 [66]. Unlike SR141716 or its bioisosteres, these compounds have a straight chain amide. The conformational freedom of this molecule is quite apparent when one attempts to position and overlay key atoms of one such molecule (**22**) with SR141716, as shown in Fig. 6**c**. These compounds were claimed to be CB1 receptor antagonists and/or inverse agonists and useful in the treatment, prevention, and suppression of diseases mediated by CB1 receptor. With this particular analog, there appear to be low-energy conformations that are not unlike SR141716, but one can also see that there are many other conformations where there is little structural analogy to SR141716. Unfortunately, no specific biological data were provided.

2.13 Aminobenzothiazoles

A recent patent application [67] included a series of aminobenzothiazoles, such as compound **23**. These compounds exhibit at least a 10-fold selectivity for the CB1 receptor. Most of them displayed modest binding affinity to CB1 receptors, the best one of the series being 0.73 μM. The 4-chlorophenyl and 2,4-dichlorophenyl represent the corresponding aromatic ring in SR141716. The benzothiazole is likely to function as the lipophilic substituent, mimicking the aminopiperidine part in SR141716. This compound is also relatively free to assume a wide variety of conformations; a fact that reveals itself when one attempts to align key atoms to the diarylpyrazole of SR141716 (Fig. 6**d**).

3 Conclusion

The importance of the endocannabinoid system continues to be demonstrated in diverse physiological roles, including food intake, energy homeostasis, immune response regulation, fertility control, and a wide variety of CNS

functions such as pain perception, learning and memory, anxiety and depression, and movement. As a result, CB1 receptor antagonists and inverse agonists have become an increasingly important pharmacological target for basic medical research and applied therapeutics. The first CB1 receptor antagonist that has gained market approval by regulatory agencies is SR141716 (Acomplia) for antiobesity/metabolic syndrome indications. Because obesity is a global epidemic that poses serious risks to public health, the market potential for Acomplia has been estimated at over US$ 1 billion annually. Other pharmaceutical interests are eager to be *fast seconds* and are rapidly deriving new antagonists for the CB1 receptor. In addition, other indications for cannabinoid antagonists are also under investigation or in clinical trials. Publications and patents are continuing to expand the chemical diversity of known CB1 receptor antagonists, which has only briefly been reviewed here. However, the structural diversity of CB1 antagonists to date appears relatively limited when compared to the wider variety of known cannabinoid agonist chemotypes (e.g., fatty acid-containing endocannabinoids [3, 68], vanilloids [69], aminoalkyl indole cannabimimetics, positive allosteric modulating indoles, and nonclassical and classical cannabinoids). Thus, one might reasonably expect to see continuing expansion of CB1 receptor ligands, including additional antagonist chemotypes and perhaps negative allosteric modulating molecules, in the near future. These new molecules may provide additional insight and therapeutic utility to a most interesting class of pharmacological agents.

Acknowledgments The authors would like to acknowledge the financial support received from the National Institute on Drug Abuse (R01 DA019217).

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Part II
Cannabinoid Receptor Biology

Cannabinoid Receptor Genetics and Evolution

Maurice R. Elphick and Michaela Egertová

Abstract The G-protein cannabinoid receptors CB1 and CB2 mediate effects of *Cannabis*-derived, synthetic and endogenous cannabinoids in humans and other mammals. Identification of the genes that encode CB1 and CB2 has facilitated utilisation of genetic strategies for pharmacological, physiological, behavioural and pathological characterisation of cannabinoid receptor function. For example, analysis of systemic or conditional CB1-knockout mice has demonstrated that CB1 is required for endocannabinoid-mediated retrograde signalling at synapses in the brain and that CB1 expression in forebrain principal neurons mediates protection against seizures. Evidence of a role for CB2 in protection against osteoporosis has emerged from analysis of polymorphisms in the human CB2 gene and analysis of bone mass in CB2-knockout mice.

CB1 and CB2 share sequence similarity indicative of a common ancestry and the gene duplication that gave rise to these cannabinoid receptors may have been concomitant with a whole-genome duplication that occurred in a common ancestor of extant vertebrates. Accordingly, CB1 and CB2 genes have also been identified in a variety of non-mammalian vertebrates (birds, amphibians and teleost fish). Another genome duplication in a common ancestor of teleost fish followed by lineage-specific gene loss probably explains the occurrence of two CB1 genes and one CB2 gene in the puffer fish *Fugu rubripes* and the occurrence of one CB1 gene and two CB2 genes in the zebrafish *Danio rerio*. Identification of CB1 and CB2 genes in non-mammalian vertebrates has enabled comparative analysis of the expression and functions of these genes in a variety of vertebrate species and several non-mammalian vertebrates are emerging as model systems for cannabinoid research.

Investigation of the phylogenetic distribution and evolutionary origins of cannabinoid receptors has been facilitated by genome sequencing. However, absence of orthologs of CB1/CB2 receptors in protostomian invertebrates (*Drosophila melanogaster, Caenorhabditis elegans*) and in the deuterostomian

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_5,

invertebrate *Strongylocentrotus purpuratus* (sea urchin) indicates that CB1/CB2-related cannabinoid receptors do not occur in the majority of invertebrates. Identification of an ortholog of vertebrate CB1/CB2 receptors known as CiCBR in the sea squirt *Ciona intestinalis* (subphylum Urochordata) indicates that cannabinoid receptors of this type originated in an invertebrate chordate ancestor of vertebrates and urochordates. Because CiCBR is the first putative cannabinoid receptor to be identified in an invertebrate, discovery of this receptor has provided a unique opportunity to investigate cannabinoid receptor function in an invertebrate species. Analysis of *Ciona* may provide an insight into the ancestral physiological roles of cannabinoid receptors in invertebrate chordates prior to the gene duplication that gave rise to CB1 and CB2 receptors in vertebrates.

Keywords Cannabinoid Receptor · Evolution · CB1 · CB2 · Invertebrate · Fish · Amphibian · Bird · *Ciona*

1 Introduction

Cannabinoid receptors can be broadly defined as proteins that mediate pharmacological effects of compounds present in extracts of the plant *Cannabis sativa*. However, it has been estimated that *Cannabis sativa* contains at least 60 'cannabinoids' (i.e., chemicals unique to *Cannabis*) [1] and therefore identifying all cannabinoid receptors in humans could be a huge task. Research on cannabinoid receptors has, not surprisingly, focused on identification of proteins that mediate the pharmacological effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the main psychoactive constituent of marijuana. Intriguingly, it appears that the effects that Δ^9-THC has on the brain are primarily mediated by just one protein, the G-protein-coupled cannabinoid receptor CB1 [2, 3]. Devane et al. (1988) first demonstrated the existence of a G-protein-coupled cannabinoid receptor in rat brain that is activated by Δ^9-THC [4] and a gene encoding this protein was identified in 1990 [5]. A second G-protein-coupled cannabinoid receptor that shares sequence similarity with the brain cannabinoid receptor was identified in immune cells in 1993 [6] and with this discovery the brain receptor was named CB1 and the immune cell receptor was named CB2.

Importantly, endogenous cannabinoids (endocannabinoids) that act as ligands for CB1 and CB2 were identified in the 1990s and these include anandamide and 2-arachidonoylglycerol (2-AG) [7]. Research on the physiological roles of endocannabinoids in humans and other mammals is now a major field of research, which is beyond the scope of this review. The primary focus of this review will be to describe the genes that encode CB1 and CB2 in humans and other mammals and then to assess the phylogenetic distribution and functions of CB1 and CB2 receptors in non-mammalian vertebrates. In addition, we will discuss the evolutionary origins of CB1/CB2-type receptors in invertebrates, focusing on CiCBR, a CB1/CB2-like receptor in the sea squirt

Ciona intestinalis and the first putative cannabinoid receptor to be identified in an invertebrate [8].

The phylogenetic distribution and evolutionary origins of cannabinoid receptors and the endocannabinoid signalling system has been discussed in detail in several recent review articles [9–13]. Here we have focused specifically on CB1 and CB2 receptors in vertebrates and CB1/CB2-related cannabinoid receptors that have been identified in invertebrate species. We have restricted our survey to CB1/CB2-related receptors because at present these are the cannabinoid receptors for which there is most information available, particularly at a molecular level and from an evolutionary perspective. However, in doing so we do not intend to imply that these are the only cannabinoid receptors in mammals or in other animals. Indeed there is good evidence that other cannabinoid receptors exist in mammals [14]. In particular, the *orphan* G-protein-coupled receptor GPR55 [15] has recently been identified as a cannabinoid receptor and preliminary supporting data have been published in abstracts [16, 17]. A survey of the phylogenetic distribution of GPR55 could be carried out presently because the sequence of this receptor is known [15]. However, this would be premature prior to publication of the first paper(s) describing the pharmacological properties of GPR55 as a cannabinoid receptor.

In addition to non-CB1/CB2 receptors that are activated by *Cannabis*-derived compounds such as Δ^9-THC or structurally related synthetic analogs, we must also give consideration to proteins, in addition to CB1 and CB2, which are activated by endocannabinoids. Here the concept of a *cannabinoid receptor* becomes blurred because whilst CB1 and CB2 receptors are activated by Δ^9-THC and by endogenous cannabinoids such as anandamide and 2-AG, there are other proteins that are activated by endogenous cannabinoids but not by Δ^9-THC or vice versa. For example, the ion channel TRPV1 is activated by anandamide but not by Δ^9-THC [18] whereas the ion channel ANKTM1 is activated by Δ^9-THC but not by endocannabinoids [19]. Thus a cannabinoid receptor is not necessarily an endocannabinoid receptor and an endocannabinoid receptor is not necessarily a cannabinoid receptor. As the molecular and pharmacological properties of non-CB1/CB2 cannabinoid/endocannabinoid receptors are characterised in more detail, a survey of the phylogenetic distribution and evolutionary origins of these proteins will become of greater interest.

2 CB1 Cannabinoid Receptor

2.1 The Human CB1 Cannabinoid Receptor Gene

The human CB1 gene was first identified in 1990 [20], based on its sequence similarity with the CB1 cannabinoid receptor discovered in rat brain [5] and is located on chromosome 6 at locus q14-q15 (NCBI Entrez Gene 1268) [21]. The 472 amino acid residue CB1 receptor protein is encoded by a single exon with 5′

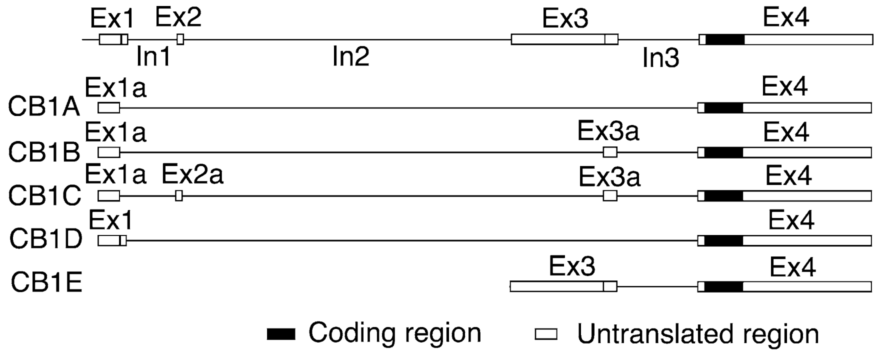

Fig. 1 Human CB1 cannabinoid receptor gene. The diagram shows the structure of the human CB1 gene, comprising three non-coding exons (Ex1–3), one coding exon (Ex4) and three introns (In1–3), and alternatively-spliced mRNA transcripts (CB1A, CB1B, CB1C, CB1D, CB1E) that have variable 5′ untranslated regions. Reproduced, with modifications, from Zhang et al. (2004) *Mol Psychiatry* 9(10):916–31

and 3′ untranslated regions (UTRs). Moreover, recent detailed analysis of the human CB1 gene [22] has revealed that there are at least three non-coding exons located 5′ to the protein-coding exon and CB1 gene transcripts are subject to alternative splicing, giving rise to CB1 mRNAs with variable 5′ UTRs (see Fig. 1). The functional significance of these mRNA variants is as yet unknown, but it is suggested that sequence variation may, for example, influence mRNA stability, subcellular localisation, or translational efficiency [22].

Polymorphisms of the CB1 gene in humans have been identified [23] and evidence of an association of particular variants with schizophrenia [24, 25], drug addiction [26, 27] and eating disorders [28] has been reported. A detailed discussion of the findings of these studies is beyond the scope of this article but can be found in two recent reviews [29, 30].

2.2 CB1 Cannabinoid Receptor Genes in Other Mammals

The CB1 cannabinoid receptor was first identified in rat [5] and is encoded by a gene located on chromosome 5 at locus q21 (NCBI Entrez Gene 25248). The rat CB1 gene comprises two exons, with a single non-coding exon located 5′ to an exon encoding the 473 amino acid residue CB1 receptor protein (NCBI Entrez Gene 25248) [5]. The CB1 gene in mouse is located on chromosome 4 (locus A5; NCBI Entrez Gene 12801) [31] and, like the rat CB1 gene, comprises two exons with a non-coding exon located 5′ to an exon encoding a 473 amino acid residue CB1 receptor protein [32, 33]. Thus, unlike the human CB1 gene, which comprises at least three non-coding exons, both the rat and mouse CB1 genes appear to have just a single 5′ non-coding exon. In addition, the CB1 protein-coding exon in rat and mouse has a much shorter 3′ UTR than the human CB1 protein-coding exon [32, 33].

Mice in which the CB1 gene has been ‘knocked out’ have been generated independently in at least three laboratories [2, 3, 34]. A consistent finding in these studies is that the well-known pharmacological effects of Δ^9-THC on wild-type mice, formalised in the so-called tetrad assay [35], are absent in CB1-knockout mice, indicating that the CB1 receptor is primarily responsible for mediating the effects of Δ^9-THC on the brain. Moreover, physiological and behavioural analysis of CB1-knockout mice has provided important new insights into CB1 receptor function in mammals, which include roles in learning and memory [36–40], analgesia [41], appetite regulation [42–44], neuroprotection [45] and endocannabinoid-mediated retrograde signalling at synapses [46–50].

In addition to systemic knockout of CB1 expression, selective deletion of CB1 gene expression in subpopulations of neurons in the brain has been accomplished using the Cre/loxP recombination system [34]. In particular, using this approach, it has been demonstrated that CB1 receptors in forebrain principal neurons mediate protection against kainate-induced seizures [34]. Development of such ‘conditional’ CB1-knockout mice is an important new strategy for determination of the relative contribution of CB1 receptors in different neuronal populations in physiological and behavioural processes.

In addition to the CB1 genes identified in human and model laboratory species (rat, mouse), partial CB1 gene sequences have also been determined for large number of other mammalian species [51]. These CB1 gene sequences were obtained as part of an investigation of the phylogenetic relationships of 64 placental mammals and two marsupials and the CB1 gene was one of several genes that were selected as suitable for this molecular phylogenetic study (i.e., >200 bp exon and 80–95% nucleotide identity between mouse and human orthologs). Further information on the mammalian CB1 genes continues to emerge as genome sequences for a variety of species are determined (e.g., chimpanzee, bovine, dog, macaque). Moreover, comparative analysis of CB1 gene structure and expression in mammals may reveal differences in CB1 function in different mammalian species.

2.3 CB1 Cannabinoid Receptor Genes in Non-mammalian Vertebrates

The extant vertebrates that are most closely related to mammals are birds and reptiles. At present nothing is known about CB1 genes in reptiles but CB1 genes have been sequenced in birds. A CB1 cDNA has been sequenced in the zebra finch *Taeniopygia guttata* and encodes a 473 amino-acid protein that shares 91% sequence identity with the human CB1 receptor [52, 53] and more recently Soderstrom (unpublished data; GI:19172409) has obtained a partial CB1 cDNA sequence from chicken (*Gallus gallus*). Sequencing of the chicken genome [54] has provided further data and analysis of genomic sequence data reveals that the chicken CB1 gene is located on chromosome 3 and encodes a

473 amino-acid protein (GI:84579889), which shares 98% sequence identity with the zebra finch CB1 receptor and 92% sequence identity with human CB1 receptor. With the availability of chicken genomic sequence data there is now an opportunity to analyse, in detail, the structure of the CB1 gene in this species in comparison with mammalian CB1 genes. For example, as in mammals, the protein-coding region of the chicken CB1 gene is located on a single exon; however, it remains to be determined if one or more exons encoding UTRs are located 5′ or 3′ to the protein-coding exon.

An amphibian CB1 receptor cDNA sequence was first identified in the rough skinned newt *Taricha granulosa* by Soderstrom et al. [55] and encodes a 473 amino-acid protein that shares 83% sequence identity with human CB1. More recently, a cDNA encoding a CB1 receptor was sequenced in the African clawed 'toad' *Xenopus leavis* and found to encode a 470 amino-acid protein, which shares 82% sequence identity with both human CB1 and *Taricha* CB1 [56]. A CB1 cDNA has also been sequenced in another frog species, the edible frog *Rana esculenta* (Meccariello et al., unpublished data; GI:78321980) and encodes a 462 amino-acid protein that shares 87% sequence identity with *Xenopus* CB1 and 82% sequence identity with human CB1. Furthermore, sequencing of the *Xenopus tropicalis* genome (see http://www.jgi.doe.gov/) will provide opportunities to investigate the structural organisation of the CB1 gene in an amphibian species in comparison with CB1 genes in other vertebrates.

The first CB1-type cannabinoid receptors to be identified in a non-mammalian vertebrate were FCB1A and FCB1B, two CB1 genes in the puffer fish *Fugu rubripes* [57]. FCB1A encodes a 468 amino-acid protein that shares 72% sequence identity with human CB1, whilst FCB1B encodes a 470 amino-acid protein that shares 66% and 59% sequence identity with FCB1A and human CB1, respectively. Thus, FCB1B is more divergent than FCB1A, by comparison with human CB1. The occurrence of two CB1-type genes in this fish species may at first sight seem surprising because in all mammalian species that have been analysed (see above) only one CB1-type receptor gene is present. However, genome sequencing has revealed that the occurrence of two orthologs of genes that exist as single-copy genes in mammals is a common feature in *Fugu* [58]. This is thought to be a consequence of a whole-genome duplication that occurred in an ancestor of *Fugu* and other teleost fish [59]. Consistent with the occurrence of two CB1 genes in *Fugu*, genes encoding orthologs of FCB1A and FCB1B also occur in another puffer fish species, *Tetraodon nigroviridis* [60]. In this species, CB1A is a 468 amino-acid protein (GI:47224365) that shares 95% sequence identity with *Fugu* CB1A and CB1B is a 474 amino-acid protein (GI:47230064) that shares 91% sequence identity with *Fugu* CB1B. However, preservation of a second CB1 gene following genome duplication may not have occurred in all teleost fish. For example, analysis of the genome sequence of the zebrafish *Danio rerio* reveals the presence of just one CB1 gene (GI:56316162), which encodes a 475 amino-acid protein that is more closely related to FCB1A (83% identity) than FCB1B (64% identity). This suggests loss of a second CB1-type gene in the fish lineage that gave rise to *Danio rerio*. These data indicate that amongst teleost fish there

may be considerable diversity in the occurrence and functions of CB1-type genes, which makes analysis of CB1 receptor function in fish of particular interest (see below). It remains to be determined, however, if the occurrence of either one or two CB1 genes in teleost fish follows a phylogenetic pattern. To address this issue more fish species will need to be analysed. Thus far only partial CB1 gene sequences have been determined for two other fish species, the goldfish *Carassius auratus* [61] and the rainbow krib *Pelvicachromis pulcher* [62], and in both cases the gene encodes a protein that is more closely related to FCB1A than FCB1B. Therefore, based on the data available at present, it seems likely that whilst a FCB1A-like receptor probably occurs throughout all teleost fish, a second FCB1B-like receptor may only occur in a subset of teleost fish lineages.

2.4 CB1 Cannabinoid Receptor Function in Non-mammalian Vertebrates

A detailed review of the physiological roles of CB1 in mammals is beyond the scope of this article and can be found in several recent articles [12, 63–65] and in other parts of this volume. The great majority of what is known about CB1 function in mammals is based on studies of rats and mice and, to a lesser extent, humans. Thus far, relatively little attention has been given to comparative analysis of CB1 function in mammals and identification of differences in the roles of CB1 in different mammalian species. This will be an interesting area of investigation for the future. In particular, comparative analysis of the distribution of CB1 in the brain may provide evidence of differences between mammalian species in the functions of the CB1 receptor.

Analysis of CB1 receptor distribution and function in non-mammalian vertebrates is beginning to provide important information that is of generic interest. In particular, non-mammalian vertebrates such as the chick and the zebrafish *Danio rerio* are emerging as useful models to investigate CB1 function. Furthermore, comparative analysis of CB1 in vertebrates is providing an insight on the functions of this receptor that are conserved in vertebrates and hence which may be evolutionary ancient.

One non-mammalian species that has been a focus of particular attention for cannabinoid research is the zebra finch *Taeniopygia guttata*. This species was selected as a model system to investigate the role of brain cannabinoid receptors in learning because, in a manner analogous to human language acquisition, male zebra finches learn a song pattern during juvenile development. The existence of cannabinoid receptors in the zebra finch brain was first established using radioligand binding assays and then a cDNA encoding a CB1-type cannabinoid receptor was cloned and sequenced [52]. Pharmacological characterisation of the cloned CB1 receptor revealed that it has similar properties to mammalian CB1 receptors [53]. Analysis of the expression of CB1 in zebra finch revealed that it is highly expressed in brain, with little or no expression in other

tissues analysed (e.g., liver, heart, lung, muscle, intestine, ovary, testis). Moreover, analysis of the pattern of CB1 mRNA and CB1 protein expression in the zebra finch brain revealed expression in regions of the telencephalon known to be involved in song learning and production [52, 66]. Consistent with these data, a cannabinoid receptor agonist was found to cause inhibition of song production in adult birds, as well as causing a decrease in locomotor activity [53]. Moreover, exposure to cannabinoids during vocal development in juveniles had an inhibitory effect on song learning [67, 68]. The physiological role of endocannabinoids in regulation of song learning and song production remains to be investigated in detail, although preliminary data indicate that in adult birds endocannabinoids may be involved in mediating the reduction in singing that occurs with limited food availability [66].

An avian species where the role of cannabinoid receptors in mechanisms of learning and memory has also been investigated is *Peocile atricapilla*, the black-capped chickadee [69]. Here intrahippocampal infusion of a CB1 receptor antagonist was found to cause enhanced long-term memory of the location of a hidden food reward. However, when the location of the food was changed, treated birds showed impairment in remembering a new location. Interestingly, studies on CB1-knockout mice have demonstrated that whilst CB1 is involved in extinction of aversive memories, it is not involved in memory extinction in an appetitively motivated learning task [70]. Therefore, there may be differences in the role of CB1 receptors in the avian and mammalian brain with the respect to memory extinction processes and further comparative studies are now required to assess the generality of the data obtained so far.

Another bird species that has been a focus of attention recently is, not surprisingly, the chicken *Gallus gallus*. Studies carried out during the 1970s demonstrated that Δ^9-THC causes tonic immobility and protection against seizures in chickens [71–73], indicating the presence of cannabinoid receptors in the avian brain and revealing similarities with the effects of Δ^9-THC on mammals. More recently, the pharmacological properties of chicken brain CB1 receptors have been investigated and found to be similar to CB1 receptors in mammalian brain [74]. Moreover, the expression of CB1 during chick brain development has been examined using mRNA in situ hybridisation methods [75]. Interestingly, CB1 expression occurs in the first-born neurons of the chick CNS (the projection neurons of the hindbrain) and then subsequently occurs in the earliest born neurons of the peripheral nervous system followed by other differentiating neuronal populations. Thus, CB1 expression follows neuronal differentiation and it is thought that CB1 may be involved in control of axonal outgrowth in new-born neurons [75]. More specifically, the expression of CB1 during development of the chick visual system has been investigated using immunocytochemical techniques [76]. Further studies are now required to analyse in detail the distribution of the CB1 receptor throughout the adult brain in chicken and in other avian species.

Several studies carried out prior to the discovery of CB1 receptors reported the pharmacological and behavioural effects of cannabinoids on frogs [77–80],

indicating the existence of cannabinoid receptors in amphibians. However, the first study to investigate the functions and properties of CB1 in an amphibian species involved analysis of the rough skinned newt *Taricha granulosa* [55]. The pharmacological properties of *Taricha* brain CB1 receptors were found to be similar to mammalian brain CB1 receptors. Moreover, analysis of the behavioural actions of cannabinoid receptor agonists revealed an inhibitory effect on spontaneous locomotor activity and courtship clasping behaviour. The effect of cannabinoids on newt locomotor activity is of particular interest because it is consistent with cannabinoid-induced hypomobility observed in mammals and birds. It suggests similarities in the organisation of CB1 expression in neural pathways that control locomotor activity in amphibians, birds and mammals. Recently, the expression of CB1 in the *Taricha* brain has been examined using mRNA in situ hybridisation methods [81]. A widespread pattern of expression was observed, with CB1 mRNA detected in telencephalon (olfactory bulb, the pallium and amygdala), diencephalon (preoptic area and thalamus), mesencephalon (tegmentum and tectum) and in the hindbrain (cerebellum and stratum griseum). These findings are largely consistent with a previous study investigating CB1 mRNA expression in *Xenopus leavis* [56]; however, there are several specific differences between *Taricha* and *Xenopus,* which are discussed in detail by Hollis et al. [81] and which indicate that there is lineage-specific variation in patterns of CB1 expression in amphibians. This is not surprising given that anurans (*Xenopus*) and urodeles (*Taricha*) are thought to have diverged from a common ancestor about 250 million years ago [82]. Nevertheless, the overall pattern of CB1 mRNA expression in amphibian brains correlates quite well with CB1 mRNA expression in mammalian brains, indicating that at least some of the functions of CB1 in the brain may have been conserved throughout the tetrapods.

The distribution of CB1 in the brain of *Xenopus leavis* has also been investigated using antibodies to the N-terminal region of rat CB1 and the pattern of immunostaining observed correlates quite well with the distribution of CB1 mRNA expression, with immunolabelled cells and/or fibres in the olfactory bulbs, dorsal and medial pallium, striatum, amygdala, thalamus, hypothalamus, mesencephalic tegmentum and cerebellum [83]. A subsequent study reported detection of CB1-like immunoreactivity in the dorsal and central fields of the *Xenopus* spinal cord, regions that correspond to laminae I–IV and X of the mammalian spinal cord [84]. This is of interest because it correlates with the distribution of CB1 in the rat spinal cord [85], perhaps indicating evolutionary conservation of a role for CB1 receptors in regulation of nociceptive processing.

Several studies reporting the actions of cannabinoids in fish species were published in the 1970s, including effects on fighting behaviour, development and synaptic transmission [86–88]. More recently, non-mammalian CB1 receptors were first identified in the puffer fish *Fugu rubripes*, but little is known about the expression of FCB1A and FCB1B in this species. RT-PCR analysis reveals that both receptors are highly expressed in *Fugu* brain, with lower level expression in ovary, testis and spleen; no expression was detected in liver,

kidney, or intestine [57]. These data are of interest because they indicate that the expression profile of CB1, with high levels of expression in the CNS and lower level expression in non-neural tissues, is an evolutionarily conserved feature that dates back at least as far as the common ancestor of teleost fish and mammals. Moreover, a recent study has provided the first insight into the temporal and spatial patterns of CB1 mRNA expression in the CNS of a fish species, the zebrafish *Danio rerio* [89]. CB1 mRNA expression is first observed quite late in development, at about 24 h postfertilisation, in cells located in the presumptive preoptic area of the diencephalon. At 48 h, expression is observed in the telencephalon, the hypothalamus, the tegmentum and the hindbrain (ventral to the cerebellum). In the adult zebrafish brain (see Fig. 2), CB1 mRNA expression is particularly abundant in the anterior region of the telencephalon and in the periventricular medial zone and central zone of the dorsal telencephalon. In the diencephalon, CB1 mRNA expression is evident in the hypothalamus and posterior tuberculum, whilst in the mesencephalon CB1-expressing cells are present in the torus longitudinalis [89]. What can we learn from these neuroanatomical expression data for CB1 in the zebrafish? Lam et al. (2005) suggest that the high levels of CB1 expression in telencephalon indicates a role for CB1 in cognitive processes, whilst expression in the periventricular medial zone may be indicative of a role in neurogenesis. Experimental manipulation of CB1 expression and activity in zebrafish is now required to investigate these hypotheses. One interesting feature of the pattern of CB1 mRNA expression in adult zebrafish brain is the lack of expression in the cerebellum because this is a brain region in which high levels of CB1 expression are found in mammalian brains [90, 91]. Further studies are now required to establish to what extent the pattern of CB1 expression in zebrafish brain is representative of other teleost species (see below).

Analysis of distribution of CB1 mRNA expression in the developing CNS of *Danio* has provided a valuable initial insight into CB1 function in fish [89]. However, detailed analysis of CB1 protein distribution in the brain of zebrafish and other teleosts is also needed as a basis for determination of the physiological roles of this receptor in fish. We have investigated CB1 expression in teleost fish by immunocytochemical analysis of goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) brains using antibodies to the C-terminal 13 amino-acid segment of the rat/human CB1 receptor, which have been used previously for analysis of CB1 expression in rat brain [90]. Surprisingly, no immunoreactivity was detected in the brains of either fish species examined. One possible explanation for this finding is that sequence differences between fish and mammalian CB1 receptors preclude detection of fish CB1 receptors with antibodies to a mammalian CB1 C-terminal protein sequence. In support of this notion, the C-terminal residue of fish CB1 receptors is a valine whereas in mammals and birds it is a leucine. To address this issue we have synthesised a peptide comprising the C-terminal 13 amino acids of the *Fugu* CB1A receptor (TMSVSTETSAEAV) and tested its reactivity with antibodies to the corresponding region of the rat/human CB1 receptor

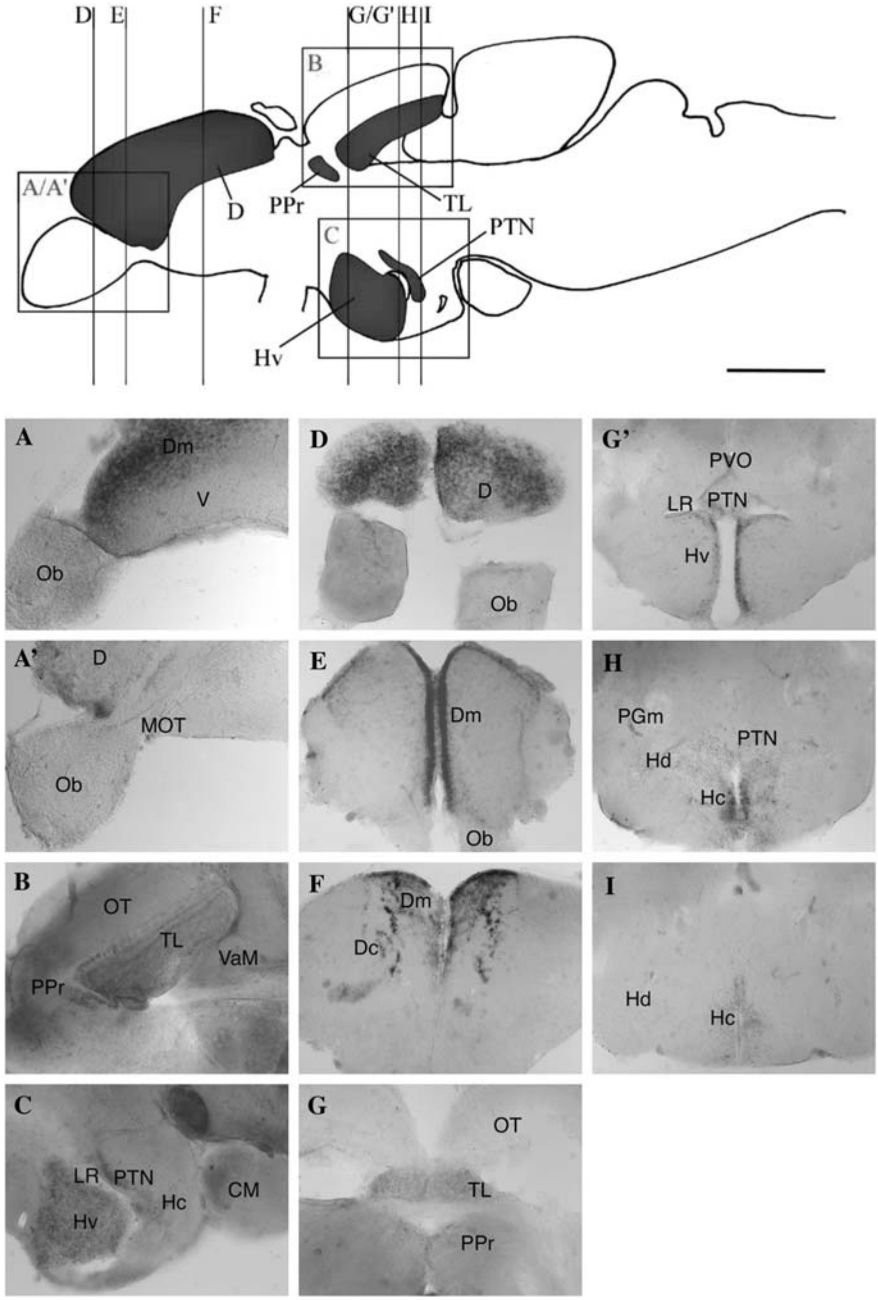

Fig. 2 Distribution of CB1 mRNA expression in the adult brain of the zebrafish *Danio rerio*. An overview of CB1 expression (shown in blue) is illustrated in the diagram. Boxes labeled in red depict sagittal views of the regions shown in panels **a–c** (**a and a′** denote two different sections of the same area) while vertical lines represent the plane of transverse sections shown

(TMSVSTDTSAEAL). Using a dot-blot assay, we found that the *Fugu* CB1A peptide was not recognised by antibodies to the rat CB1 peptide. Furthermore, the *Fugu* peptide did not block immunostaining in rat brain revealed by our antibodies to rat CB1 when tested at a concentration (20 μM) at which staining is completely blocked by the rat CB1 C-terminal peptide. Thus, it appears that a single amino acid substitution at the C-terminus of teleost CB1 receptors is sufficient to render these receptors non-reactive with antibodies to C-terminal region of the rat/human CB1 receptor. These data illustrate the importance of careful characterisation of antibodies to mammalian proteins when using them for analysis of non-mammalian species.

Recently, Cottone et al. (2005a,b) have investigated the distribution of CB1 in fish brains using antibodies targeting the N-terminal region of the rat CB1 receptor and antibodies targeting a larger segment of the C-terminal region of CB1 than our C-terminal antibodies discussed above [61, 62]. Western blot analysis of brains from the cichlid *Pelvicachromis pulcher* revealed the presence of a protein that was recognised by both the N-terminal and C-terminal directed CB1 antibodies and that had a molecular mass similar to rat CB1. Immunocytochemical analysis of CB1 expression in the cichlid brain revealed immunostained neurons and/or fibres in several brain regions including the telencephalon, the periventricular preoptic nucleus, the lateral infundibular lobes of the hypothalamus, the pretectal central nucleus and the posterior tuberculum. Comparison of these data with those reported by Lam et al. (2005) is complicated by the use of different species and different experimental methods for revealing CB1 expression. Therefore, it would be useful to see a direct comparison of CB1 mRNA and CB1 protein expression performed on

Fig. 2 (Continued) in panels **d–i**. Scale bar = 500 μm beneath the schematic. The olfactory bulbs are largely devoid of CB1 expression (panels **a, a′**). In the forebrain, CB1 is expressed predominantly in the dorsal telencephalon, pretectum, hypothalamus and posterior tuberculum (panels **b, c**) while expression in the midbrain is restricted to the torus longitudinalis (panel **b**). In transverse sections, CB1 expression is detected in anterior regions of the dorsal telencephalon with faint staining in the olfactory bulbs (panel **d**). Slightly posterior, strongly stained CB1-expressing cells are restricted to the ependymal layer, which extends to the edges of the everted dorsal telencephalon (panel **e**). At midtelencephalic levels (panel **f**), CB1-expressing cells are detected in the medial and central zones of the dorsal telencephalon. Further back (panel **g**), weak CB1 expression is localised in the torus longitudinalis and the periventricular pretectum. In the hypothalamus (panels **g′, h, i**), CB1 expression is restricted to the periventricular zones. Very faint labeling is detected in the corpus cerebelli. (Abbreviations: CM, corpus mamillare; D, dorsal telencephalon; Dc, central zone of dorsal telencephalon; Dm, medial zone of dorsal telencephalon; Hc, caudal zone of periventricular hypothalamus; Hd, dorsal zone of periventricular hypothalamus; Hv, ventral zone of periventricular hypothalamus; LR, lateral recess; MOT, medial olfactory tract; Ob, olfactory bulb; OT, optic tectum; PGm, medial preglomerular nucleus; PPr, periventricular pretectal nucleus; PTN, posterior tuberal nucleus; PVO, paraventricular organ; TL, torus longitudinalis; V, ventral telencephalon; VaM, medial division of valvula cerebelli). Reproduced, with modifications, from Lam et al. (2006) *Neuroscience*, 138:83–95 (*See* Color Plate 1).

the brains of both of these fish species. Nevertheless it is clear from the data currently available that there are similarities in the patterns of CB1 mRNA expression in zebrafish brain and CB1 protein expression in neuronal somata of the cichlid brain. Interestingly, however, whilst CB1 expression was not observed in the zebrafish cerebellum [89], CB1 immunoreactivity was observed in a subset of granule cells and Purkinje cells in the cichlid brain [62]. Therefore, it is important that these initial studies are followed up by further studies to establish whether or not there are fundamental differences in the patterns of CB1 expression in different teleost species. This will be of particular interest in those teleosts such as the puffer fishes *Fugu rubripes* and *Tetraodon nigroviridis* in which two CB1 receptor genes occur.

As more detailed information on the anatomical distribution of CB1 receptors in the CNS of teleost fish becomes available, experimental analysis of CB1 function in these animals will become more tractable. Recently, Valenti et al. (2005) have investigated a possible role for the endocannabinoid system in control of food intake in the goldfish *Carassius auratus,* measuring the effect of food deprivation on endocannabinoid levels in the telencephalon and testing the effect of the endocannabinoids on food intake [92]. Further studies are needed to investigate the role of the CB1 receptor in this system and this could be accomplished using CB1 antagonists.

One aspect of fish physiology where the role of CB1 receptors has been investigated in considerable detail is in the visual system or more specifically in the retina. The distribution of the CB1 receptor in the fish retina was first investigated as part of a wider study analysing the retina of several vertebrate species, including rhesus monkey, rat, mouse, chick, tiger salamander and goldfish [93]. Cannabinoid receptor agonists were found to cause a substantial reduction in the amplitude of voltage-activated L-type calcium currents in bipolar cells. Subsequent studies have investigated the electrophysiological effects of cannabinoids on several retinal cell types, using the goldfish and the tiger salamander as model systems [94–96].

As discussed above, CB1 genes have yet to be identified in cartilaginous or agnathan fish. Nevertheless, a recent study on an agnathan fish, the lamprey *Lampetra fluviatilis*, has provided an important insight into the role of a putative CB1 receptor in this species and more generally a potential role for the CB1 receptor in regulation of spinal locomotor neural activity in vertebrates. Kettunen et al. (2005) found that a CB1 receptor antagonist caused a decrease in fictive locomotor rhythmic neural activity whilst activation of metabotropic glutamate receptors caused an endocannabinoid-mediated increase in activity [97]. Collectively, the data obtained in this study are consistent with a model in which glutamatergic activation of motoneurons triggers formation of endocannabinoids, which then act as retrograde messengers to cause CB1-mediated inhibition of glycine release from inhibitory interneurons that synapse with contralateral motoneurons and excitatory interneurons. Thus, the endocannabinoid system appears to be involved in modulation of locomotor pattern generation in the lamprey and it is possible that it has a

similar role in other vertebrates, including mammals. This study illustrates very nicely how research on cannabinoid receptor function in non-mammalian vertebrates is beginning to provide novel insights into the physiological roles of the endocannabinoid system.

3 CB2 Cannabinoid Receptor

3.1 The Human CB2 Cannabinoid Receptor Gene

The human CB2 cannnabinoid receptor gene is located on chromosome 1 at locus p36.11 (NCBI Entrez Gene 1269) and comprises an exon encoding the 360 amino acid residue receptor protein and a 5′ exon encoding a UTR [6, 98]. Interestingly, a recent study indicates that polymorphisms in the human CB2 gene are associated with postmenopausal osteoporosis [98]. Another recent study has obtained evidence that a polymorphism in the CB2 gene is associated with reduced endocannabinoid-mediated immunomodulation and may be a risk factor for autoimmune disorders [99].

3.2 CB2 Cannabinoid Receptor Genes in Other Mammals

The rat CB2 cannabinoid receptor gene is located on chromosome 5 at locus q36 (NCBI Entrez Gene 57302) and the 410 amino acid residue receptor protein is encoded by three exons [100]. The mouse CB2 cannabinoid receptor gene is located on chromosome 4, locus D3 (NCBI Entrez Gene 12802) and comprises an exon encoding a 347 amino acid residue receptor protein and a 5′ exon encoding a UTR [101]. Genome-sequencing projects have also identified CB2 cannabinoid receptor genes in chimpanzee (GI:55586475), dog (GI:73950616) and cow [GI:76611561).

Development of CB2 gene-knockout mice has been important for determination of the physiological and pathological roles of the CB2 receptor. Buckley et al. first investigated the consequences of CB2 gene-knockout and found an absence of the immunomodulatory effects of cannabinoids that are observed in wild-type animals [102]. In particular, Δ^9-THC inhibits helper T-cell activation through macrophages derived from wild-type, but not from knockout mice, indicating that this effect is mediated by the CB2 receptor. More recently, analysis of CB2-knockout mice has provided evidence of a role for the CB2 receptor in protection against osteoporosis because these animals have a decreased bone mass compared to wild-type littermates [103]. These findings are consistent with the association of CB2 polymorphisms and postmenopausal osteoporosis reported by Karsak et al. [98]. It is likely that further analysis of CB2-knockout mice will lead to the identification of other physiological processes and disorders involving the CB2 receptor.

3.3 CB2 Cannabinoid Receptor Genes in Non-mammalian Vertebrates

Analysis of the chicken genome reveals the presence of a CB2 gene encoding a putative 346 amino acid residue receptor protein (GI:50759962) but as yet no cDNA sequence data are available. Similarly, BLAST analysis of the genome of the amphibian *Xenopus tropicalis* (http://genome.jgi-psf.org/Xentr4) reveals the presence of CB2 gene but determination of the full receptor sequence will require assembly of genomic shotgun sequence data and corroboration from cDNA sequencing.

Elphick (2002) was the first to report the identification of a CB2 gene in a non-mammalian vertebrate having analysed the genome of the puffer fish *Fugu rubripes* for the presence of cannabinoid receptor genes [104]. The two CB1 receptor genes (FCB1A, FCB1B) originally discovered by Yamaguchi et al. [57] and a single CB2 gene were identified [104]. The *Fugu* CB2 gene comprises an exon that encodes a putative 379 amino acid residue receptor, which shares 50% amino acid identity with human CB2 and 47% amino acid identity with human CB1. Moreover, sequence alignment and construction of phylogenetic trees using the neighbour-joining method demonstrated that the *Fugu* CB2-like receptor is an ortholog of mammalian CB2 receptors [104].

As discussed above, it is thought that the occurrence of two CB1 genes in *Fugu* may be a consequence of a genome duplication that occurred in an ancestor of teleost fish 300–450 million years ago [12, 104]. Therefore, the presence of just one CB2 gene in *Fugu* may reflect loss of a duplicate gene. Consistent with this view, analysis of the genome of another puffer fish species (*Tetraodon nigroviridis*) also indicates the presence of just one CB2 gene (GI:47196823). Intriguingly, however, analysis of the genome of the zebrafish *Danio rerio* reveals the presence of two CB2 genes: one located on chromosome 12 (NCBI Entrez Gene 560848; GI:68370508) and another located on chromosome 16 (NCBI Entrez Gene 405900; GI:47085729). Further studies are now required to investigate the *Danio* CB2 genes in more detail. Nevertheless, what the data available indicate is that in the fish lineage that gave rise to puffer fishes (*Fugu*, *Tetraodon*) two CB1 genes were retained and one CB2 gene was lost following genome duplication in an ancestor of teleost fish. In contrast, the data indicate that in the fish lineage that gave rise to the zebrafish *Danio rerio* two CB2 genes were retained and one CB1 gene was lost. The functional significance of these differences is unknown (see below); nevertheless, our findings provide the basis for further investigation of cannabinoid receptor gene diversity and function in teleost fish.

3.4 CB2 Cannabinoid Receptor Function in Non-mammalian Vertebrates

As CB2 cannabinoid receptor genes have only been identified in non-mammalian vertebrates relatively recently [104], it is not surprising that at present little is

known about CB2 receptor function in non-mammalian vertebrates. Indeed, to the best of our knowledge there are no published studies that have specifically investigated CB2 function in non-mammalian vertebrates. The zebrafish *Danio rerio* would be an attractive model system for future studies and it will be of particular interest to compare the expression and functions of the two CB2 genes identified here in the genome of this species (see above).

4 The Evolution of CB1 and CB2 Cannabinoid Receptors in Vertebrates

Analysis of non-mammalian vertebrates (see above) indicates that the occurrence CB1 and CB2 genes can be traced back at least as far as the common ancestor of osteichthyes (which include teleost fish) and tetrapods (amphibians, reptiles, birds, mammals). It remains to be determined, however, whether or not CB1 and CB2 receptors also occur in the chondrichthyes (cartilaginous fish such as sharks and rays) and agnathans (hagfish and lampreys). If, as suggested above and previously [12], the duplication event that gave rise to CB1 and CB2 genes from a common ancestral gene was concomitant with a whole-genome duplication, then we need to consider the timing when this genome duplication took place. This is a controversial issue and a consensus has yet to be reached about the number and timing of genome duplications during the early vertebrate evolution [59]. However, one school of thought is that two genome duplications occurred in quick succession (perhaps separated by only 10 million years) very early in vertebrate evolution. If this is correct, then duplicated genes arising from the genome duplications may have been present in the ancestor of all extant vertebrates and accordingly all extant vertebrates may have CB1 and CB2 genes. It will be of particular interest, therefore, to analyse the occurrence and characteristics of cannabinoid receptors in the most primitive extant vertebrates—lampreys and hagfish (Agnatha). Important in this regard is the recent study discussed above [97], which provided pharmacological evidence for the presence of CB1-type cannabinoid receptors in the spinal cord of the lamprey *Lampetra fluviatilis*. However, definitive conclusions must await gene or genome sequencing and importantly the National Human Genome Research Institute (http://www.genome.gov) recently announced that the sea lamprey *Petromyzon marinus* is one of eighteen organisms given priority status for genome sequencing.

5 The Evolutionary Origin of CB1/CB2-Related Cannabinoid Receptors in Invertebrates

If, as discussed above, CB1 and CB2 cannabinoid receptor genes evolved following duplication of an ancestral gene during early vertebrate evolution then it is likely that the invertebrate ancestor of vertebrates had a single CB1/

CB2-related cannabinoid receptor gene. Therefore, in tracing the evolutionary origins of CB1/CB2-related cannabinoid receptors, our attention should first focus on extant invertebrate chordates (cephalochordates and urochordates), which are more closely related to the vertebrates than other invertebrates. The cephalochordates are exemplified by the lancelets (Amphioxus), which include several species belonging to the genus *Branchiostoma*. To the best of our knowledge, there have been no studies that have specifically investigated the occurrence of cannabinoid receptors in *Branchiostoma*. However, following a proposal to sequence the genome of a cephalochordate species [105] the DOE Joint Genome Institute (http://www.jgi.doe.gov) has recently obtained whole-genome sequence data for the Florida lancelet *Branchiostoma floridae*. Therefore, analysis of the occurrence of cannabinoid receptor genes in this cephalochordate species will soon be practicable.

The urochordates include species such as the sea squirt *Ciona intestinalis* and the genome of this species has been sequenced [106]. An important resource associated with the *Ciona* genome project is a database of partial (EST) and full-length cDNA sequences [107] and analysis of this database led to the identification of a cDNA encoding an ortholog (CiCBR) of vertebrate CB1/CB2 cannabinoid receptors [8]. CiCBR is a 423 amino-acid protein that shares 28% and 24% sequence identity with human CB1 and CB2, respectively. Moreover, phylogenetic analysis of CiCBR using multiple sequence alignment methods demonstrated that CiCBR is an ortholog of vertebrate CB1 and CB2 receptors [8]. Thus, CiCBR is not more closely related to either CB1 or to CB2 but is positioned outside the CB1 and CB2 clades of a phylogenetic tree (see Fig. 3). These data indicate that the common ancestor of CiCBR and vertebrate CB1/CB2 cannabinoid receptors predates the gene (genome) duplication event that gave rise to CB1-type receptors and CB2-type receptors in vertebrates, supporting the hypothesis (see above) that CB1 and CB2 receptors evolved during early vertebrate evolution.

CiCBR is encoded by a gene comprising nine exons: exon 1 encodes a 5′ UTR and the N-terminal part of the receptor, exons 2–7 encode internal protein sequences, exon 8 encodes the C-terminal part of the receptor and a 3′ UTR and exon 9 encodes a 3′ UTR [8]. The structure of the CiCBR gene with multiple protein-coding exons is unusual by comparison with most CB1 and CB2 genes in vertebrates, where the receptor protein is typically encoded in a single exon (see above). The only currently known exception to this is the rat CB2 gene, which comprises three protein-coding exons [100].

The discovery of CiCBR in *Ciona intestinalis* is important because it is the first putative cannabinoid receptor gene to be identified in an invertebrate species. Moreover, the discovery of CiCBR indicates that the ancestry of CB1/CB2-related cannabinoid receptors can be traced back at least as far as the chordate ancestor of vertebrates and urochordates. It remains to be determined, however, whether the ancestry of CB1/CB2-related cannabinoid receptors extends further back to a common ancestor of chordates and non-chordate invertebrate phyla.

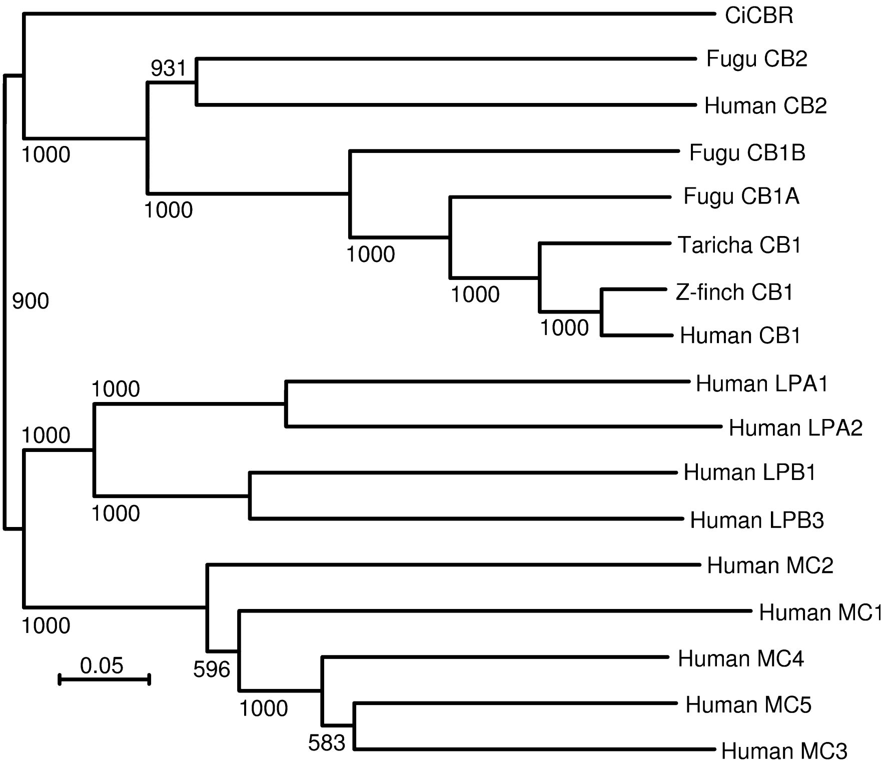

Fig. 3 CiCBR: An ortholog of vertebrate CB1 and CB2 receptors in the urochordate *Ciona intestinalis*. The phylogenetic tree shows that CiCBR is an ortholog of vertebrate cannabinoid receptors because it is positioned together with vertebrate cannabinoid receptors in a clade supported by a bootstrap score of 900/1000. However, CiCBR is positioned outside the CB_1 and CB_2 clades of the tree, indicating that the common ancestor of CiCBR and vertebrate cannabinoid receptors predates a gene duplication event that gave rise to CB1-type and CB2-type receptors. Human lysophospholipid receptors (LPA1, LPA2, LPB1, LPB3) and human melanocortin receptors (MC1–MC5) are shown as outgroups because they are more closely related to cannabinoid receptors than all other known G-protein-coupled receptors in humans. The tree was constructed using the neighbour-joining method with bootstraping (1000 bootstrap trials). Reproduced from Elphick et al. (2003) *Gene* 302(1–2):95–101

The non-chordate invertebrates that are most closely related to the chordates are the hemichordates and echinoderms and collectively the chordates, hemichordates and echinoderms belong to an assemblage of animals known as deuterostomes. Therefore, analysis of hemichordates and echinoderms could reveal whether or not the ancestry of CB1/CB2-related cannabinoid receptors extends back to the common ancestor of all deuterostomes. The hemichordates were until recently a relatively poorly studied phylum but this situation is changing and there is growing interest in these animals in the fields of developmental biology and comparative genomics [108]. Moreover, the acorn worm *Saccoglossus kowalevskii* was recently approved by the National Human Genome Research Institute

(http://www.genome.gov) for genome sequencing and therefore investigation of the occurrence of cannabinoid receptor genes in this species may soon be feasible.

The echinoderms include animals such sea urchins, sea cucumbers, starfish and brittlestars. The sea urchin *Strongylocentrotus purpuratus* has been studied in detail by Herbert Schuel and colleagues to investigate the occurrence and possible physiological functions of cannabinoid receptors in this species [109]. In particular, cannabinoid-binding sites have been detected on sea urchin sperm [110] and it is thought that endocannabinoids acting on these receptors may be involved in prevention of polyspermy by blocking the acrosome reaction [111]. However, the molecular identity of the protein that confers cannabinoid sites on sea urchin sperm is unknown and it is not known if CB1/CB2-related cannabinoid receptors occur in sea urchins and other echinoderms. Importantly, however, the genome of the sea urchin *Strongylocentrotus purpuratus* has recently been sequenced by the Baylor College of Medicine Human Genome Sequencing Center (http://www.hgsc.bcm.tmc.edu/projects/seaurchin). We have analysed the *Strongylocentrotus purpuratus* genome sequence data and interestingly we have not found any genes encoding proteins that are orthologs of CiCBR and the vertebrate CB1/CB2 cannabinoid receptors. Similarly, our previous analysis of the genome sequences of protostomian invertebrate species such as *Drosophila melanogaster* and *Caenorhabditis elegans* revealed that orthologs of the vertebrate cannabinoid receptors are not present in these species [12]. Therefore, what the available data indicate is that CiCBR/CB1/CB2-related cannabinoid receptors originated in an ancestral chordate species and accordingly receptors of this type are found in vertebrates and invertebrate chordates (e.g., *Ciona*) but not in other deuterostomian invertebrates (echinoderms) or in protostomian invertebrates (e.g., arthropods, nematodes). Therefore, by comparison with other G-protein-coupled receptors, which are found throughout the animal kingdom (e.g., muscarinic acetylcholine receptors), CB1/CB2-related cannabinoid receptors have a much more restricted phylogenetic distribution and appear to be unique to chordates. These findings are important because they identify those invertebrate species that can be utilised as model systems to investigate the physiological roles of CB1/CB2-related cannabinoid receptors (see below). However, as discussed above in the introduction, other as yet undiscovered cannabinoid receptors might exist and have a wider phylogenetic distribution than CB1/CB2-related receptors. In particular, it will be of interest to investigate the molecular identity of proteins that confer cannabinoid-binding sites in tissues from non-chordate invertebrates such as echinoderms [110], molluscs [112] and cnidarians [113].

6 CiCBR: A Model Ancestral Cannabinoid Receptor?

Discovery of CiCBR in the sea squirt *Ciona intestinalis* [8] has provided a unique opportunity to investigate the physiological roles of a putative cannabinoid receptor in an invertebrate species. Moreover, comparative analysis of

CiCBR function in *Ciona* and CB1/CB2 function in vertebrates may provide an insight into the roles that an ancestral-type cannabinoid receptor may have had in a common ancestor of the chordates.

Ongoing studies are investigating the pharmacological properties of CiCBR (K. Soderstrom, M. Egertová & M.R. Elphick; unpublished data). Furthermore, we have developed specific antibodies to CiCBR, which recognise a membrane protein in homogenates of adult *Ciona* that has a molecular mass (~47 kDa) consistent with that expected for CiCBR based on its amino acid sequence. We are currently using these antibodies to analyse the expression of CiCBR in adult *Ciona* and, intriguingly, our data indicate that it is particularly abundant in the cerebral ganglion (M. Egertová and M.R. Elphick, unpublished data). Ongoing identification of cell types expressing CiCBR will provide a foundation for investigation of the physiological roles of this receptor in *Ciona*.

Discovery of CiCBR by Elphick et al. [8] has also attracted interest from other research groups and expression of CiCBR in adult *Ciona* tissues has been examined using PCR [114]. Evidence of CiCBR expression in cerebral ganglion, heart, intestine, ovaries, pharynx, stomach and testis was obtained; however, detailed anatomical analysis of these tissues is required to determine the presence and localisation of CiCBR mRNA and/or protein. The effect of a cannabinoid receptor agonist (HU210) on a behavioural response in adult *Ciona* has also been investigated. Tactile stimulation of the inhalent siphon in *Ciona* causes siphon closure and Matias et al. investigated the effect of HU210 on the time taken for siphon reopening [114]. Interestingly, treatment with HU210 appears to cause lengthening of the time taken for siphon reopening. However, the maximum effect was not observed until 150 min after drug application. Further studies are now required to investigate a physiological basis for these behavioural/pharmacological observations. In particular, it remains to be determined whether or not the effect of HU210 is mediated by CiCBR or by other as yet unidentified proteins. Moreover, if the effect of HU210 is mediated by CiCBR, the effect of HU210 on siphon re-opening needs to be investigated in relation to the pattern of CiCBR expression in adult *Ciona*. In particular, the long delay (~150 min) before a maximal effect of HU210 is observed invites explanation.

7 General Conclusions and Future Perspectives

Genetic techniques have been crucial in determining the physiological and pathological roles of CB1 and CB2 cannabinoid receptors in humans and other mammals. In particular, development and analysis of CB1- and CB2-knockout mice have provided and continue to provide new insights into the physiological roles of cannabinoid receptors. Looking ahead, the development of conditional knockout mouse models for CB1 [34] will enable more detailed

dissection of the role this receptor in different aspects of CNS function and in other aspects of mammalian physiology. The identification of genes encoding novel cannabinoid receptors (e.g., GPR55) will open up entirely new avenues for research on the cannabinoid system and for development of new drugs that selectively target different receptor types.

Comparative analysis of non-mammalian vertebrates has demonstrated that CB1- and CB2-type cannabinoid receptors can be traced back at least as far as the common ancestor of teleosts and tetrapods. Genome sequencing of primitive vertebrate species such as the lamprey will reveal whether the occurrence of these two receptor types extends back as far as the common ancestor of all extant vertebrates. The discovery of CB1 and CB2 receptors in a variety of non-mammalian vertebrate species has also opened up new opportunities for exploiting these animals as model systems for cannabinoid research. However, at present, virtually nothing is known about CB2 receptor expression and function in non-mammalian vertebrates and this is surely an area of research where new data will soon emerge. The discovery of novel physiological roles for the CB2 receptor in processes such as regulation of bone formation will act as stimulus for analysis of CB2 function in non-mammalian vertebrate systems.

Quite a lot is now known about CB1 expression in non-mammalian vertebrates but still relatively little is known about function. Moreover, if we are to acquire a detailed understanding of how the CB1 receptor system has evolved in the vertebrates, many more species will need to be analysed than the few selected species investigated so far. Comparative neuroanatomy is fraught with difficulties in identifying patterns of gene/protein expression that are representative of any particular clade. Nevertheless, the publications that have appeared so far, reporting either CB1 mRNA or protein expression in the brains of selected fish, amphibian and avian species, hint at some general themes. As more species are analysed it may be possible to model how the CB1 receptor system has evolved in parallel with changes in vertebrate brain architecture that have taken place over several hundred million years of evolutionary time. In so doing, our understanding of the functions of the CB1 receptor will be enhanced and the consequences of therapeutic manipulation of CB1 function more deeply appreciated. In particular, it will be of interest to investigate the neurotransmitter-phenotype of neurons expressing CB1 in non-mammalian vertebrates. In the mammalian forebrain, CB1 is expressed at very high levels in GABAergic neurons and at lower levels in glutamatergic principal neurons [12, 34]. Therefore, it will be interesting to determine if similar associations are found for CB1 receptor expression in the telencephalon of non-mammalian vertebrates.

Investigation of the evolutionary origins of the CB1/CB2-type cannabinoid receptors has been made possible by genome sequencing of selected invertebrate species. The discovery of CiCBR in the urochordate *Ciona intestinalis* and the absence of CB1/CB2-receptor orthologs in other invertebrate species examined so far indicate that CB1/CB2-related cannabinoid receptors originated in an ancestor of the chordates. Therefore, as discussed above, analysis of cannabinoid receptor function in invertebrate chordates such as *Ciona* is of particular

interest. Looking ahead, analysis of the *Branchiostoma* genome may lead to the discovery of a second invertebrate CB1/CB2-related cannabinoid receptor. Moreover, as interest in endocannabinoid signalling becomes more widespread it is likely that new types of cannabinoid/endocannabinoid receptors will be discovered in invertebrates, some of which may turn out to be unique to particular groups of invertebrates.

Acknowledgments The original work of the authors reported in this article was supported by a grant (S19916) from the BBSRC (UK).

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Addendum

While this book was in press, the following papers relevant to this chapter have been published:

Egertová, M. and Elphick, M.R. (2007) Localization of CiCBR in the invertebrate chordate *Ciona intestinalis*: evidence of an ancient role for cannabinoid receptors as axonal regulators of neuronal signalling. J. Comp. Neurol. 502, 660–672.
Elphick, M.R. (2007) BfCBR: a cannabinoid receptor ortholog in the cephalochordate Branchiostoma floridae (*Amphioxus*). Gene 399, 65–71.

Part III
Cannabinoid Receptor Molecular Pharmacology

Cannabinoid Receptor Signal Transduction Pathways

Emma Scotter, Scott Graham, and Michelle Glass

Abstract The cannabinoid receptors CB1 and CB2 each couple to intracellular G proteins in order to transduce agonist binding into a cellular response. Signalling by the two receptors can differ markedly, as indeed can signal transduction through each individual receptor in response to various ligands. The divergence of signalling is regulated at various stages—from G protein coupling to activation of effectors, and in many cases appears to be cell type specific. This review will initially examine the ability of CB1 and CB2 to activate G proteins, and then look at downstream pathways, both G protein dependent and independent, that are activated by these receptors.

Keywords GPCR · Signalling · Stimulus trafficking · ERK · Krox 24 · cAMP

1 G-Protein-Coupled Receptors: A Brief Overview

G-protein-coupled receptors (GPCRs) represent the largest superfamily of cell-surface receptors and play a key role in cellular responsiveness to ligands as diverse as hormones, neurotransmitters, odorants and even light [1]. While non-canonical pathways for GPCR signalling are now emerging [reviewed in 2, 3], the traditional view is that GPCRs transduce ligand binding into a cellular response primarily through the activation of GTP-binding proteins (G-proteins).

Heterotrimeric G-proteins comprise an alpha subunit, whose subtype is critical in determining the downstream effectors linked to a particular GPCR, and a beta–gamma ($\beta\gamma$) dimer, which is also able to activate effectors with some subtype specificity [4]. Under basal conditions the Gα subunit is GDP bound and tightly associated with the $\beta\gamma$ dimer. However, activated GPCRs can

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_6,

induce the Gα subunit to exchange GDP for GTP and release the βγ dimer, enabling signalling through both the free Gα-GTP and βγ components [4].

Many GPCRs have the ability to couple to various different Gα subtypes, thus eliciting varying responses. The specificity of Gα coupling may be determined by the particular ligand bound to the GPCR, a phenomenon known as 'agonist-directed trafficking of signalling' or 'stimulus trafficking' [5, 6]. This may indicate that binding of that particular ligand induces a GPCR conformation which favours activation of a specific Gα subtype. Alternatively a GPCR which is precoupled to a particular Gα subtype may present a favourable binding conformation for that ligand. There is evidence for both lines of thought, with the latter view allowing for the phenomenon of 'sequestration' of G proteins. It has been shown that high affinity pre-coupling of inactive GPCRs, exemplified by CB1, to a particular Gα-GDP subtype may limit signalling of other GPCRs which couple to the same Gα subtype [7].

These general features of GPCR signalling are shared by the CB1 and CB2 cannabinoid receptors; although such a generalized analysis belies the complexity of the specific outcomes of such signalling.

2 Stimulus Trafficking of Cannabinoid Response

The two-state model for GPCR activation supposes that GPCRs exist in only two conformations, active (R*) or inactive (R). However, Glass and colleagues (1999) first described the ability of various cannabinoid agonists to induce specific conformations of the CB1 receptor which favour coupling to a specific G protein, that is, the receptor is capable of 'stimulus trafficking' [8]. They showed, through reconstitution activity assays, that while the synthetic ligand HU-210 could induce maximal activation of both Gαi and Gαo by CB1, the ligand WIN55,212-2 was a full agonist only for Gαi activation of CB1. These two ligands may therefore stabilize two different active conformations of CB1, one which can maximally activate both Gαi and Gαo, and one which has limited efficacy in activating Gαo [8].

CB1 coupling to Gαs may also show stimulus trafficking. Bonhaus et al. have shown that the potency of CP-55,940 and HU-210 in stimulating adenylate cyclase is greatly enhanced in the presence of forskolin, while WIN-55212-2 stimulation of adenylate cyclase shows no synergy with forskolin [9]. They suggest this may reflect the different binding properties of CP-55,940 and HU-210 compared to WIN-55212-2, causing conformational differences in the receptor [10]. This may then result in trafficking of Gαs activation of different adenylate cyclase isoforms, of which type I is atypical, showing no synergy with forskolin [9, 11].

The report of a WIN55,212-2-specific coupling of CB1 to Gαq highlights again the importance of stimulus trafficking with regard to understanding cannabinoid signalling [12]. Various studies had previously reported no change

in inositol phosphate production following CB1 receptor stimulation by a variety of ligands, however these studies did not investigate WIN55,212-2 effects [8, 13, 14]. CB1 appears to couple with far lower affinity to Gαq than Gαi/o as pertussis toxin inactivation of the latter G proteins greatly enhances the ability of CB1 to elevate intracellular calcium via Gαq [12].

In the case of CB2, Shoemaker and colleagues recently demonstrated differential potency, but not efficacy, of activation of three distinct signalling pathways by different ligands in CHO–CB2 cells [15]. While the synthetic agonist CP-55,940 and endocannabinoid noladin ether both potently inhibited adenylate cyclase activity, they less potently activated the extracellular signal regulated kinase (ERK) 1/2 pathway. In contrast, the structurally similar endocannabinoid, 2-arachidonoyl glycerol, was found to activate the ERK 1/2 pathway most potently [15]. However, the specific G proteins involved in these varied responses remain to be investigated as CB2 has only been shown to couple strongly to Gαi [8, 16].

Recent data suggest that stimulus trafficking may extend to regulation of not only G-protein type, but also subtype. Three subtypes of Gαi exist [1–3] which share over 80% homology [17]. Howlett and colleagues have shown that the $G\alpha i_1$ and $G\alpha i_2$ subtypes interact with the CB1 receptor at its third intracellular loop, while $G\alpha i_3$ may interact with the proximal portion of the C-terminus [18]. These Gαi subtypes may be differentially activated by cannabinoid ligands as $G\alpha i_1$ and $G\alpha i_2$, but not $G\alpha i_3$, are typically activated by the same ligands [19].

These data strongly support a model whereby agonists are able to stabilize different receptor conformations which favour specific G protein couplings, extending to the level of specific G protein subtypes. This may then determine the potency of an agonist in eliciting a particular response.

3 Cannabinoid Receptor Signalling

3.1 Adenylate Cyclase

Both CB1 and CB2 receptors couple to the inhibition of cyclic adenosine monophosphate (cAMP) accumulation in a pertussis toxin sensitive manner [14]. Consistent with this, both receptors couple with high affinity to the G protein Gαi as determined by [35S]GTPγS binding in in situ reconstitutions [8]. The Km values for incorporation of [35S]GTPγS upon receptor activation are 28.3 ± 2.2 nM and 39.7 ± 3.6 nM for CB1 and CB2 respectively [8] . The activation of the G protein Gαi by CB1 or CB2 allows the dissociation of the Gαi subunit from the βγ dimer. GTP-bound Gαi may then associate with its effector molecule adenylate cyclase, inhibiting adenylate cyclase-mediated cAMP production. This in turn reduces the activation of cAMP-dependent protein kinase A (PKA) which requires cAMP in order to dissociate from its autoinhibitory subunits [reviewed in 20].

While CB1 and CB2 show comparable affinity for Gαi, differences in signal transduction between the two cannabinoid receptors become evident upon treatment of CB receptor-expressing cells with pertussis toxin. This toxin induces ADP-ribosylation of both Gαi and Gαo [21], preventing their GTP-binding and dissociation from βγ in response to receptor occupancy.

In the absence of functional Gαi, a stimulatory effect of CB1 upon cAMP accumulation is unveiled which appears to operate through Gαs [22]. Pertussis toxin ablation of functional Gαi may mimic the situation in vivo where CB1 interacts heterodimerically with D2 dopamine receptors. This heterodimer shows a 'switch' in coupling from inhibition of adenylate cyclase by either GPCR alone, to stimulation when both receptors are activated simultaneously [22, 23] This activation of Gαs stimulates adenylate cyclase to increase cAMP production, leading to PKA disinhibition and cAMP response element (CRE)-mediated transcription [24].

While Gαi sequestration by D2 dopamine receptors, independent of their activation state, has been suggested to be responsible for CB1 coupling to Gαs [25], this is at odds with studies which show that CB1 itself is able to sequester Gαi/o proteins from a myriad of other GPCRs [7]. Alternatively, the elegant studies of O'Dowd and colleagues suggest that oligomeric association of GPCRs may modulate the active conformation of each receptor monomer [26]. Hetero-oligomerization of CB1 with D2 could potentially stabilize the CB1 receptor in a conformation different from that in which it exists as a homo-oligomer even with the same ligand bound. Therefore, stimulus trafficking of signalling may be subject to a further level of regulation by an oligomeric partner, in this case unveiling a signalling pathway unavailable to the activated monomer alone.

A recent in vivo study examining the effects of the CB1 agonist CP-55,940 suggests that the contribution of Gαs-coupling to CB1 signalling in the brain may be greater than previously expected. Andersson and colleagues (2005) found that the motor depression elicited by CP-55,940 acted through phosphorylation, at the PKA site, of DARPP-32 in inhibitory GABAergic medium spiny striatal neurons [27]. They suggest that a Gαs-coupled increase in cAMP may be responsible for phosphorylation of DARPP-32 which then inhibits protein phosphatase-1 to activate these inhibitory striatal neurons. Furthermore, pharmacological inactivation or knockout of the D2 dopamine receptor prevented the ability of the CB1 agonist to cause motor depression, implicating the CB1/D2 heterodimer in the response to CP-55,940 [27].

These findings challenge the traditional view that cannabinoid agonists suppress locomotor activity solely through presynaptic inhibition of neurotransmitter release. The ability of cannabinoids, through a D2 dopamine receptor-dependent 'switch' to Gαs-coupling, to increase the excitability of inhibitory neurons is now being realized.

In contrast to the CB1 receptor, to date CB2 has not shown functional coupling to Gαs, with pertussis toxin treatment of CB2-transfected CHO cells showing no stimulatory effect on cAMP accumulation [22].

3.2 Calcium Channels

CB1 signalling, through pertussis toxin sensitive G proteins, has been shown to inhibit Ca^{2+} influx via L-, N-, P/ Q-, and R-type voltage-sensitive Ca^{2+} channels [28–30]. The differential inhibition of these subtypes of Ca^{2+} receptor is evident in cerebellar granule cells which express each subtype at the synaptic bouton [31]. It has been found that in these cells the synthetic cannabinoid WIN55,212-2 preferentially inhibits N-type Ca^{2+} channel influx, but also strongly inhibits P/Q- and R-type Ca^{2+} channel influx [32].

It has been proposed that endocannabinoids acting at presynaptic CB1 receptors are responsible for depolarization-induced suppression of excitation (DSE) or inhibition (DSI) via this inhibition of Ca^{2+} influx (See Fig. 1) [33, 34]. Endocannabinoids released by depolarized postsynaptic neurons may act as retrograde ligands at presynaptic CB1 receptors. The resulting inhibition of presynaptic Ca^{2+} channels may serve to limit either excitatory or inhibitory outputs to the postsynaptic neuron by inhibiting Ca^{2+}-dependent neurotransmitter release from that particular terminal [33]. Recent evidence suggests that 2-arachidonylglycerol (2-AG) may be a key player in retrograde signalling in the hippocampus as inhibitors of the monoacylglycerol lipase which specifically metabolises 2-AG enhanced DSI in hippocampal pyramidal neurons [35].

In addition to the immediate inhibitory effects of CB1 activation on plasmalemmal voltage-gated Ca^{2+} channels, a mechanism for long-term modulation of intracellular calcium levels has recently been proposed. The inhibition of cAMP accumulation through CB1 activation of Gαi may preclude phosphorylation of a ryanodine receptor (RyR) which modulates intracellular calcium channels. By preventing phosphorylation of the RyR, calcium release from intracellular stores is reduced [36]. This mechanism may have particular relevance to CB1 neuroprotection in excitotoxic cell death which has been shown to develop more consistently following intracellular calcium dysregulation than transient extracellular calcium influx after injury [37].

Despite the trend for cannabinoid administration to decrease intracellular calcium levels through Gαo and possibly Gαi, as mentioned, sporadic reports of transient calcium increases following CB-receptor stimulation also exist. As discussed, there is persuasive evidence of a Gαq linkage for CB1, which is associated with administration of WIN55,212-2, but not other cannabinoid agonists such as HU-210, CP-55,940, 2-AG, methanandamide, or cannabidiol [12]. WIN55,212-2-stimulated CB1 coupling to Gαq appears to link CB1 to intracellular calcium release through the classical PLCβ/ IP3 pathway. This involves phospholipase activity at the plasma membrane which releases the second messenger inositol triphosphate (IP3), a ligand for IP3 receptors which gate the release of calcium from intracellular stores [38].

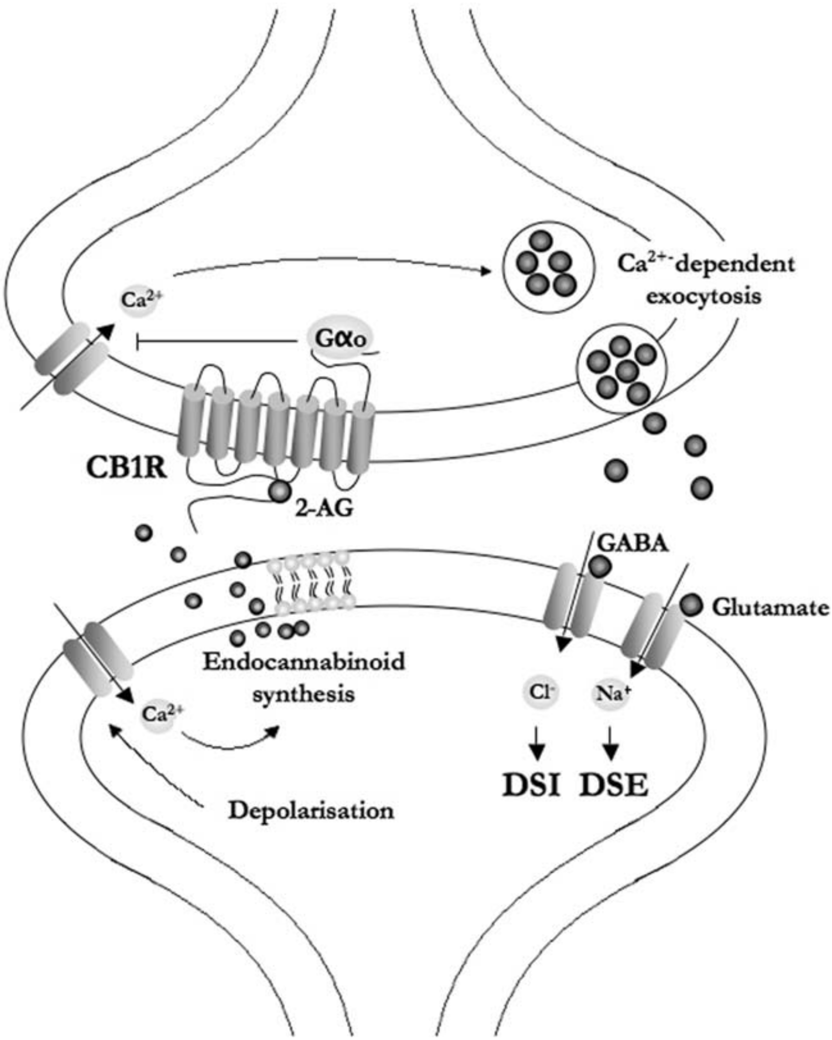

Fig. 1 Depolarization-induced suppression of excitation or inhibition. Depolarization of the postsynaptic neuron (bottom) causes calcium influx, initiating endocannabinoid synthesis from membrane lipids. The 2-AG released acts on presynaptic terminal CB1 receptors (top) which inhibit calcium-dependent neurotransmitter release through Gαo

3.3 Inwardly Rectifying Potassium Channels

CB1, but not CB2, is coupled to the activation of G protein coupled inwardly rectifying potassium channels (GIRKs) [14]. By stimulating K^+ influx through these channels in neurons, CB1 receptor signalling tends to hyperpolarize presynaptic terminals. This in turn has been suggested to reduce voltage-sensitive

Ca^{2+} influx, in addition to the direct inhibition of Ca^{2+} channels discussed previously, reducing Ca^{2+}-mediated exocytosis of neurotransmitter [39]. Interestingly, although the CB2 receptor has been shown not to couple to GIRK or calcium-channel activation in AtT20 cells [30], some activation of GIRK currents by CB2 was seen, albeit inconsistently, in a *Xenopus* oocyte expression system [16].

3.4 Ceramide: Sphingomyelin Hydrolysis and Synthesis De Novo

The CB1 receptor has also been shown to couple to the hydrolysis of sphingomyelin, a ubiquitous component of the outer leaflet of the plasma membrane, in primary astrocytes and glioma cells, but as yet not in primary neurons [40–42]. Sphingomyelin hydrolysis releases phosphorylcholine and ceramide, which functions as a second messenger and may be involved in linking CB1 activation to the ERK 1/2 pathway via mobilization of Raf-1 [42] (see Fig. 2). A transient elevation of ceramide levels occurs rapidly following CB1 stimulation and is associated with increased glucose consumption in the astrocytic cultures in which it is observed [42].

It has been suggested that sphingomyelinases may be activated in a G protein-independent fashion through an adaptor protein, factor associated with neutral sphingomyelinase activation (FAN), which binds the CB1 C-terminus. FAN binds the tumor necrosis factor receptor (TNFR) which in turn activates sphingomyelinases [43]. Additionally FAN can be coimmunoprecipitated with the CB1 receptor and its inactivation prevents CB1-mediated ceramide generation [44].

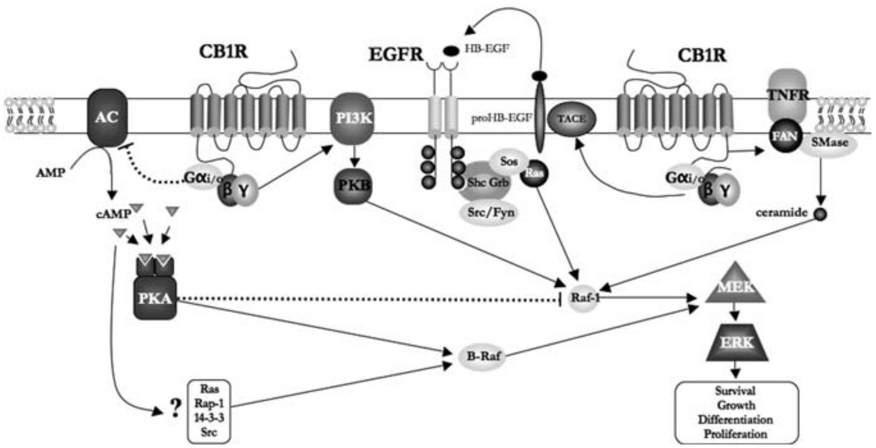

Fig. 2 Mechanisms of CB1 activation of the Raf/MEK/ERK pathway: CB1 may couple to the activation of ERK through several different routes including cAMP-dependent pathways, PKA-dependent pathways, the activation of PI3K/PKB, transactivation of tyrosine receptor kinases, or ceramide synthesis

In addition to the acute production of ceramide through sphingomyelin hydrolysis, CB1 or CB2 activation may also be responsible for ceramide synthesis *de novo* in transformed cell lines [41]. Chronic stimulation of C6 glioma cells causes a sustained accumulation of ceramide which, like transient ceramide elevation, activates ERK 1/2 through Raf-1 [40]. However, this chronic CB receptor stimulation is associated with the initiation of apoptosis, rather than an enhancement of primary oxidative processes as seen in astrocytic acute ceramide elevation. With respect to glioma, CB2-selective agonists may be of some therapeutic benefit in stimulating apoptosis, through ceramide synthesis, without targeting normal neuronal cells which are devoid of this CB receptor [41].

Several studies have shown that ceramide has an inhibitory influence upon the phosphatidylinositol-3-kinase (PI3K)/ protein kinase B (PKB) pathway [45–47] while activation of ERK 1/2 and p38/ c-Jun N terminal kinases (JNK) by CB1 is maintained [48]. This data supports a paradigm where PI3K/ PKB tone may influence the cellular outcome of downstream mitogen-activated protein kinase (MAPK) events indirectly, in this case favoring apoptotic processes despite the activity of ERK 1/2 which might otherwise have directed a proliferative fate.

3.5 Raf-1/MEK/ERK Cascade

Due to the importance of the Raf-1/ mitogen-activated protein kinase (MEK)/ ERK pathway in the control of many genes involved with growth, differentiation, and proliferation this pathway has attracted significant attention from the cannabinoid field. Questions that researchers have asked pertain in particular to the precise molecular mechanisms that are involved in transducing CB receptor activation of the Raf/ MEK/ ERK cascade.

Some debate has centered on the requirement for internalization of GPCRs in order to activate MEK/ERK. In early studies, dominant negative dynamin mutants which were used to prevent β2-adrenergic receptor internalization also prevented the active receptor from stimulating phosphorylation of ERK 1/2 [49]. At face value this suggests that ERK 1/2 activation has a requirement for either receptor internalization or signalling through internalization-related pathways such as β-arrestins, GRKs, or other internalization adaptor proteins. However it was more recently shown that a non-internalizing μ-opioid receptor was able to activate ERK 1/2 in the absence, but not in the presence of, a dominant negative dynamin mutant [50]. This dynamin-dependence of ERK activation was elegantly explained by the work of Pierce and colleagues who showed a requirement for internalization not of the GPCR in question, but of the tyrosine kinase receptor it transactivates [51]. For the CB1 receptor, Mackie and colleagues showed ERK 1/2 activation to occur following stimulation of a non-internalizing (D164N) CB1 mutant

suggesting that for the CB1 receptor also, GPCR internalization is not critical for ERK coupling [52].

Downstream of receptor activation events, several signalling pathways have been reported to couple CB receptors to activated ERK (see Fig. 2). These include (a) βγ activation of the PI3K pathway, (b) via cAMP-dependent mechanisms, or (c) via transactivation of receptor tyrosine kinases (TRK), although these routes are by no means exhaustive or exclusive of each other.

The involvement of the PI3K pathway has been demonstrated in several studies using a combination of pharmacological inhibitors of PI3K [53, 54] which subsequently prevent the activation of PKB/Akt, the principal target of PI3K signalling. However, the dependence of ERK 1/2 activation upon the PI3K/ PKB pathway may be cell specific. For instance, CB2 has been shown to activate ERK 1/2 in the HL60 cell line [55, 56], but does not activate the PI3K/ PKB pathway in these cells [48]. In contrast PI3K activation is required for CB1 activation of ERK in CHO and U373 MG cells [54]. Also both PI3K and PKB are required for CB1 or CB2 activation of ERK 1/2 in PC-3 cells [53]. Interestingly, Molina-Holgado and colleagues have demonstrated that activation of the PI3K/PKB pathway in primary cortical neurons did not result in the concomitant activation of the ERK 1/2, p38, or JNK cascades [57]. Thus, it is clear that PI3K is not the only route for CB receptor activation of ERK nor is ERK activation necessarily induced by its activity.

It has been suggested that ERK 1/2 activation by CB1 is sensitive to regulation of the cAMP/PKA system. Bouaboula et al. describe an increase in basal ERK 1/ 2 phosphorylation following the addition of cAMP analogs, although CB1 stimulation was additive with these analogs in its stimulation of ERK, suggesting that in this case the CB1 component of ERK1/2 activation occurred through an alternative pathway [58]. In contrast several groups describe systems in which increased cAMP, through either forskolin administration [59], or addition of cAMP analogs [60], inhibits the ability of CB1 to activate ERK 1/2. This may be explained by differential activation of either Raf-1 or B-Raf by cAMP-activated PKA, leading to either the inhibition or activation respectively of ERK 1/2 (See Fig. 2) [59, 61]. PKA phosphorylates Raf-1 at three sites (S43, S233, and S259), holding it in an inactive form [61, 62], and preventing subsequent activation of MEK by Raf-1. Unlike Raf-1, B-Raf lacks the inhibitory PKA phosphorylation sites present in Raf-1, and is activated by a number of cAMP-dependent routes. Therefore the relative activity and expression of these two members of the Raf family will contribute to some of the differences observed between cAMP levels and activation of the ERK cascade. For the CB2 receptor, studies in CHO cells also demonstrate an additive effect of cAMP analogues and CB2 activation, possibly implicating a disinhibition of ERK 1/2 through Raf-1 by the CB2 receptor, concurrent with B-Raf-mediated activation of ERK by cAMP [55].

Alternatively it has been suggested that CB1 may transactivate members of the TRK family leading to activation of ERK through these receptors. In N18TG2 neuroblastoma cells, the TRK involved may be the vascular

endothelial growth factor receptor (VEGF), as specific inhibition of this TRK by oxindole-1 prevents CB1 activation of ERK 1/2 [63]. However, a depression of VEGF signalling in glioma cells following cannabinoid administration has also been reported [64]. Alternatively, in a number of tumour cells transactivation of epidermal growth factor receptor (EGFR) has been demonstrated, leading to subsequent activation of SHC, and downstream activation of ERK 1/2 [65]. This study described a cannabinoid-mediated cleavage of the membrane spanning proEGF-like growth factor ligands proAmphiregulin and/or proHeparin-binding epidermal growth factor-like growth factor (proHB-EGF), at the cell surface by TNF-α-converting enzyme (TACE/ADAM17) which lead to subsequent activation of EGFR [65]. Finally, there is strong evidence for the role of the downstream Src-family kinase, Fyn, in ERK 1/2 activation, as Fyn knockout mice show no elevation in phospho-ERK levels in response to cannabinoid administration [60].

In contrast, it appears that a completely different pathway may exist in neural progenitor cells where CB1 activation leads to an inhibition of ERK1/2 activity, through inhibition of the Rap1/B-Raf/ERK pathway [66].

Taken together these studies demonstrate that there are multiple mechanisms of ERK activation following stimulation of the cannabinoid receptors. Although there are numerous contradictory reports of CB receptor-mediated signalling events and some may play more important roles than others in vivo, these studies provide an indication of the complexity of signalling that may occur at the synapse.

3.6 c-Jun N-Terminal Kinase p38 Mitogen Activated Protein Kinase

The JNK and p38 kinases are important members of the MAPK family and have been associated with growth, differentiation, and the control of cell fate and apoptosis in response to cellular stress [reviewed in 67]. Through activation of these kinases, cannabinoids have been shown to inhibit the growth of various tumour cells including leukaemia [68], neuroglioma [69, 70], and lymphoma [71] in addition to promoting apoptosis in specific immune cells [72].

Herrera and colleagues demonstrated that CB2 receptors activate p38 in human leukaemia cells following stimulation by THC, resulting in the initiation of programmed cell death [68]. The mechanism linking CB receptor-stimulated p38 activity with apoptosis is currently still under investigation, although the authors suggest a direct regulation of caspases 3 and 8 by p38, independent of an effect on mitochondrial membrane potential or permeability [68]. Experiments performed with glioma cell lines that endogenously express the CB1 receptor suggest that sustained JNK activation may also be involved in THC-induced cell death [73]. Unfortunately, apoptotic outcomes due to cannabinoid activation of the p38 and JNK pathways are not restricted to cancer cells, as in

rat primary cortical cells THC activation of JNK through CB1 also results in apoptosis [74].

The p38 and JNK MAPK pathways are also thought to play a key role in mediating inflammatory responses. Sugiura and colleagues have observed a pronounced activation of p38 and JNK in LH-60 leukemic cells following stimulation of CB2 receptors by 2-AG, a proposed novel inflammatory messenger [75]. This increases the production of interleukin-8 and macrophage–chemotactic protein which are then involved in the infiltration of inflammatory immune cells [reviewed by 75]. So while the p38 and JNK MAPKs appear to be involved in quite different processes to those associated with ERK 1/2 induction by cannabinoids, they have in common a complex and divergent signalling cascade which allows for highly specific outcomes in different cannabinoid-responsive cell types.

4 Immediate Early Genes

Several immediate early transcription factors are upregulated in specific brain regions following cannabinoid agonist administration. These include Krox 24 [60, 76] and Fos [77, 78], with both genes induced in a tissue-specific manner. In vitro studies have recapitulated some of these results observed in brain. Bouaboula and colleagues demonstrated CB1-mediated upregulation of Krox 20, Krox 24, and Jun B in human astrocytoma cells, although Fos expression was not affected [79]. CB2 activation is similarly associated with an upregulation of Krox 24, however, Fos expression is decreased by CB2 receptor agonism [55, 80].

4.1 Krox 24 and Fos Transcription Factors

The promoter regions for both the Krox 24 and Fos transcription factors contain serum response element (SRE) and CRE consensus binding sites in addition to autoregulatory binding sites for negative feedback and suppression of transcriptional activity [81, 82].

For SRE activation of Fos, phosphorylated SRF combines with a phosphorylated ternary complex factor forming the SRF–ternary complex, which then binds to the DNA consensus sites to initiate transcription [83, 84]. In contrast, CRE-mediated transcription requires binding of the CRE-binding (CREB) transcription factor [24], whose activity is regulated by the acetyltransferase activity of CREB-binding protein (CBP) [85].

The binding and activation of both SRE and CRE have been shown to involve the activation of ERK 1/2. For example, phosphorylation and targeting of the ternary complex factor Elk-1 (Ets-like protein), in response to cannabinoids, has been linked to the ERK 1/2 pathway [86]. Transient activation of the ERK 1/2 signalling pathway also induces phosphorylation of CREB and subsequently Krox 24 expression via CRE-mediated transcription [87], although

whether the THC-mediated increases in CREB seen in rat brain [88] are ERK-dependent is yet to be investigated. The extent and specificity of activation of these transcription factors is in part associated with the location of ERK within the cell. This reflects the diversity of its cytoplasmic and nuclear substrates [reviewed by 89].

Krox 24 is a zinc finger-binding transcription factor with three consensus zinc fingers which each bind three nucleotides in the recognition sequence 5′GCGGGGGCG in the promoter regions of target genes [90]. The Krox 24 gene was codiscovered by several groups searching for genes involved in growth, differentiation, and proliferation, hence the array of aliases in the literature, which include zif268 [91], Egr-1 [92], NGFI-A [93], and ZENK, which was derived from the first letter of the previous aliases. The first in vivo studies to report changes in IEG expression following cannabinoid administration reported a stimulation of Krox 24 immunoreactivity in rat striosomes following CP-55,940 administration [76].

Fos, like Krox 24, is capable of dimerization with numerous other transcription factor proteins, including the classical AP1 partners, to regulate transcription of a myriad of genes. Consensus binding sites for these Fos-containing dimers are present in the promoter regions of many genes [reviewed by 94].

A THC-induced increase in Fos immunoreactivity has been shown in the dorsal striatum and nucleus accumbens of rat by Miyamoto and colleagues, probably reflecting activation of terminal CB1 receptors in the globus pallidus or substantia nigra pars reticula [95]. Dopaminergic transmission via D1 receptors may also be involved in the cannabinoid-mediated induction of Fos as it was significantly attenuated by a D1 antagonist in this context [95]. Studies which show D1 antagonism to also attenuate both the ERK 1/2 and Elk-1 response to THC corroborate a pathway for Fos activation through ERK 1/2 and Elk-1 [86].

The dependence of striatal D1 receptors in THC-mediated ERK 1/2 and IEG responses in vivo suggests that the signalling events being measured (Fos, Krox 24, and pERK immunoreactivity) may not occur in the primary THC-responsive cells [86, 95]. Because exogenous THC administration can stimulate dopamine release in the striatum [96, 97], a strong induction of pERK, Fos, and Krox 24 through dopamine receptors may be masking weaker or more transient direct CB1-mediated effects in vivo. However, in brain slice preparations, which also overcome some of the drug distribution issues seen in vivo, 2-AG stimulates ERK 1/2 phosphorylation in hippocampal neurons within 1 min, indicative of direct signalling from CB1 [60].

4.2 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF) is an IEG which has been shown to be regulated by cannabinoids in a number of studies. Several of these demonstrate upregulation of BDNF mRNA associated with CB1 signal transduction

in vivo [60, 98, 99]; however, there have also been reports of CB1-mediated decreases in BDNF expression [100, 101], indicating a complex control system may be involved. Like c-Fos and Krox 24, the promoter region for BDNF contains a CRE sequence which underlies the dependence of BDNF expression upon cAMP levels [102].

In a kainic-acid (KA) lesion model of Huntington's disease, mice respond to KA administration with an upregulation of Krox 24, c-Fos, and BDNF, resulting in reduced severity of the lesion [103]. However, slice cultures from CB1 knockout mice lack this upregulation of BDNF in response to injury, leading to an increase in resulting lesion area which can be reversed by application of exogenous BDNF [99]. In contrast to these findings, chronic administration of THC has also been associated with depression of depolarization-induced [101] and basal [100] BDNF expression in rodent models.

However, because BDNF expression levels are highly dependent upon neuronal activity [104], it is not surprising that BDNF mRNA levels are regulated by cannabinoids, whose neuromodulatory effects may either suppress or enhance neuronal activity through DSE and DSI, respectively.

5 Conclusion

The signalling pathways evoked by cannabinoid administration and originating at either the CB1 or CB2 receptors are complex and as yet poorly understood. Mitogenic, proliferative, or even apoptotic outcomes may be seen following cannabinoid exposure. This GPCR family demonstrates the principal of 'stimulus trafficking' where G protein-coupling and activation of particular effectors is often agonist specific, leading to differential outcomes even against the same cellular background.

The various cell death/ survival signalling pathways responsive to CB receptor stimulation present the potential for cannabinoid administration for various therapeutic reasons. The upregulation of cell survival factors such as BDNF and Krox 24 may be useful in neurodegenerative disorders such as Huntington's disease. Additionally the divergence of signalling between CB1 and CB2 receptors may be exploited in the specific targeting of glioma cells using CB2-selective agonists. However, the complexity of signaling associated with CB-receptor activation must be considered fully when cannabinoids are to be used therapeutically in order that adverse events are avoided and their therapeutic potential is realized.

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Cannabinoid Agonist and Inverse Agonist Regulation of G Protein Coupling

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Abstract The CB1 cannabinoid receptor is a G-protein-coupled receptor (GPCR) that can be stimulated by cannabinoid and nonclassical cannabinoids, aminoalkylindoles, and endocannabinoids. Constitutive activity of CB1 receptors allows inverse agonists such as arylpyrazoles to modulate activity in the absence of an agonist, and can allow sequestration of Gi/o proteins to alter signal transduction involving other receptors. Agonist-stimulated activity can be noncompetitively blocked by allosteric drugs. Agonists selectively activate G proteins, and may behave as inverse agonists for certain subtypes. CB1 receptors can couple to Gs under conditions of inactivation of Gi proteins or unique states of interaction with dopaminergic receptors that might include heterodimerization.

Structure of the intracellular domains of the CB1 receptor defines the G protein selectivity and activation. Palmitoylation defines the intracellular loop 4 (IL4), and phosphorylation sites can regulate G protein coupling. The CB1 receptor IL3 is relatively long, forming an amphipathic α-helix believed to interact with G proteins. The IL4 can form an α-helical segment at the end of transmembrane (TM) 7, which in a negatively charged environment can exist as a 3_{10}-helix. The IL3 interacts with Gi1 and Gi2, whereas the IL4 interacts with Gi3 and Go. Special motifs including YXXIXXL/A, BBXB, or BBXXB, and the IL4 NPXXY$(X)_{5,6}$F are important for activity. Activation of the CB1 receptor probably involves sequential microconformational changes that may be initiated by a steric trigger as the agonist releases internal-binding energy within its binding pocket.

Keywords Anandamide · 2-Arachidonoylglycerol · CP55940 · Endocannabinoid · Immunoprecipitation · Recombinant receptors · Rimonabant · Steric trigger model · Ternary complex model

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_7,

1 Introduction

The CB1 cannabinoid receptor is a G-protein-coupled receptor (GPCR) that can be stimulated by cannabinoid and nonclassical cannabinoid ligands such as Δ^9-tetrahydrocannabinol (THC), HU210, desacetyllevonantradol, and CP55940; aminoalkylindole ligands such as WIN55212-2; endocannabinoids 2-arachidonoylglycerol, and anandamide and analogs such as (R)-methanandamide; and antagonized by arylpyrazoles such as rimonabant (also known as SR141716) (see the review by the International Union of Pharmacology Cannabinoid Receptor Committee [1]). When stimulated by agonists, the CB1 receptor activates effectors such as adenylyl cyclase and ion channels, mitogen-activated protein kinase (MAPK), and other signaling pathways using the Gi/o family and other G proteins as signal transducers. Certain antagonist ligands (prototypically rimonabant) can signal responses that are opposite those stimulated by agonists, indicating that the CB1 receptor has some constitutive activity, and thus, these ligands are referred to as inverse agonists (see review by Pertwee [2]). A recent investigation described allosteric regulation of the CB1 receptor by a novel class of ligands [3]. Research also indicates that cannabinoid receptor agonists can signal differentially to selective G proteins (see review by Howlett [4]). The present chapter examines these studies and presents some speculation regarding the molecular mechanism for the agonist-receptor-G-protein selectivity for the CB1 cannabinoid receptor.

2 Constitutive Activity of the CB1 Receptor

2.1 Mechanism for Constitutive Activity

Constitutive activity in the absence of an agonist has been described both theoretically and experimentally, and inverse agonists can diminish the constitutive activity when it is manifest [5, 6]. In recombinant expression systems for GPCRs, constitutive activity is more likely to be observed with high concentrations of exogenously expressed receptor, which drive the receptor plus G protein equilibrium reaction in the forward direction, thereby increasing the probability of a spontaneous isomerization from inactive (RG_{GDP}) to active (R*G_) forms in the absence of an agonist (see Fig. 1). Constitutive activity has also been shown for mutant GPCRs having amino acid changes that preclude important internal bonding interactions that would normally maintain the protein in the ground state until an agonist can induce a conformational change. One might infer that similar conformational changes induced by the agonist must exist as relatively transient intermediate stages in the course of the receptor–G protein activation mechanism. If a receptor were to exhibit constitutive activity in its native state, it might do so by either mechanism: high expression levels that increase the probability of precoupling to G proteins, or

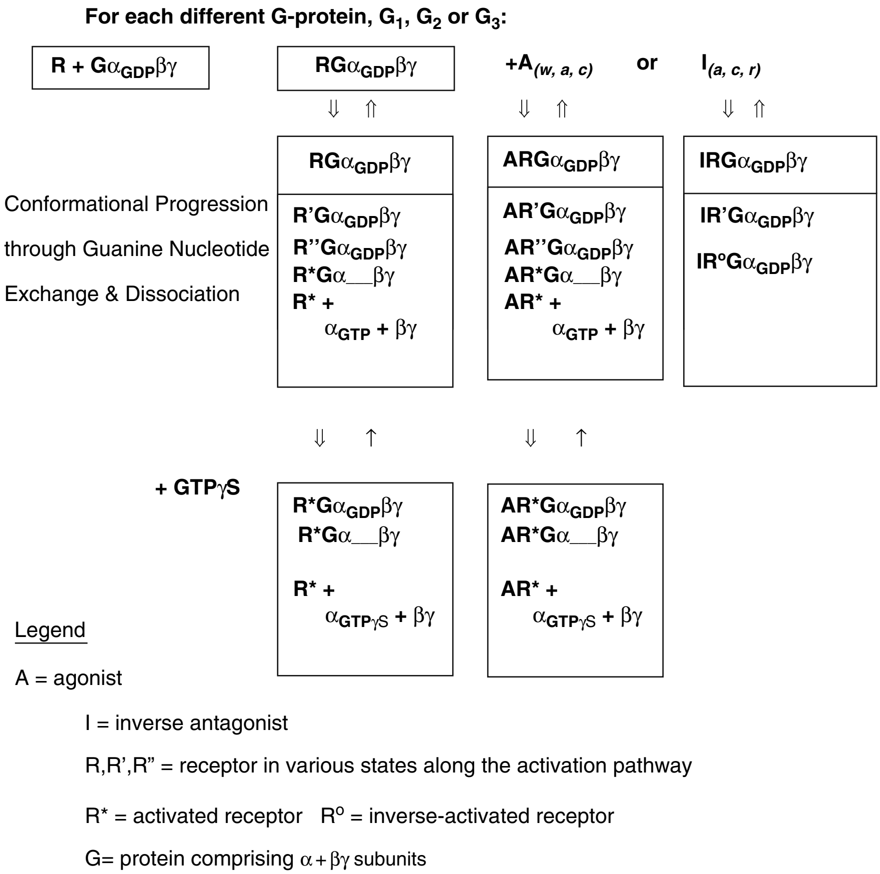

Fig. 1 Scheme depicting the principle of micro-conformational changes leading to activation or inverse activation of the CB1 receptor-G-protein (RG) complex. It is proposed that multiple, chemically distinct ligands (e.g., WIN55212-2 (***w***), cannabinoids (***c***), anandamide (***a***), rimonabant (***r***)) can bind RG with interactions at different transmembrane domains, or with different amino acid residues of the same transmembrane domains. Ligands ***w***, ***a***, and ***c*** may interact differentially with the various G proteins complexed with R. For example, agonist ***w*** may take all three G proteins RG_1, RG_2, and RG_3 completely through a progression to ***w***R^*G_1 and ***w***R^*G_2 and ***w***R^*G_3. Ligand ***a*** might be limited to taking only a single G-protein type (e.g., RG_3) all the way through the progression to ***a***R^*G_3. Ligand ***a*** may, however, take RG_1 and RG_2 only as far as ***a***$R'G_1$ and ***a***$R'G_2$, and yet may have a much greater probability of proceeding in an alternate conformational progression to R^oG_1 and R^oG_2. Similarly, ligand ***c***, may progress the RG complex to activation (e.g., ***c***R^*G_1 and ***c***R^*G_2 behaving as an agonist), and RG_3 to inverse activation (e.g., ***c***R^oG_3). For ligand ***r***, acting as an inverse agonist at all three G-protein types, the progression for different RG complexes RG_1, RG_2 or RG_3 may proceed through to R^oG_1, R^oG_2 or R^oG_3

having a tertiary structure conducive to conformational isomerization from inactive to active receptor in the absence of an agonist.

Constitutive activity has been observed for CB1 receptors, and the ligand rimonabant has been most highly studied as an inverse agonist for this receptor. MAPK basal activity was greater and adenylyl cyclase activity was reduced in Chinese hamster ovary (CHO) cells expressing CB1 receptor compared with nontransfected control cells [7]. This was easily demonstrated by the ability of rimonabant to reverse the augmented basal responses due to the CB1 receptor expression. However, these properties have been observed in native cell models [8–10] as well as recombinant cells overexpressing CB1 receptors [7, 9, 11], indicating that the agonist-independent activity is not simply an artifact of overexpression. An inverse agonist (I)-supported inactive state ($IR^{o}G_{GDP}$) was proposed by Bouaboula and colleagues [7] to describe a mechanism for the CB1 receptor to "sequester" Gi proteins. This inactive state could explain the finding that basal signal transduction through the MAPK or adenylyl cyclase pathways was blocked in the presence of rimonabant [7]. By this mechanism, inverse agonists could promote sequestration of G proteins with CB1 receptors in an inverse agonist-R^{o}-G_{GDP} complex, which would reduce the fraction of RG_{GDP} complex that could spontaneously convert to R*G (Fig. 1).

The expression of recombinant CB1 receptors in CHO cells increased basal [^{35}S]GTPγS binding to G proteins in membranes compared with wild-type CHO, and rimonabant in the 1–30 nM concentration range reversed this response [7, 12, 13]. This would suggest that the presence of CB1 receptors exerted a tonic influence on G proteins in the membranes. In studies reported by Sim-Selley and colleagues [10], higher concentrations of rimonabant were required for the inverse agonist effect in [^{35}S]GTPγS binding assays, even under assay conditions which promoted constitutively activated receptors [10].

Howlett's laboratory examined receptor–G protein interactions using the N18TG2 neuroblastoma cell model system [14]. These cells endogenously express CB1 receptors and all three subtypes of Gi. The study quantitated the Gαi and βγ proteins that coimmunoprecipitated with the CB1 receptor from a 3-[(-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) extract of N18TG2 cell membranes. CB1 receptor-Gα complexes appear to exist in detergent solution even without the addition of agonists to stabilize a ternary complex [15–18]. In detergent solution, GTPγS promoted dissociation of the G proteins from the CB1 receptor as determined by disruption of the association of Gαi proteins with immunoprecipitated CB1 receptors [14]. Gβγ was dissociated from the CB1 receptor in parallel with Gαi, supporting the notion that the heterotrimer dissociation allows release of both components of the heterotrimer from the receptor. This finding indicates that the RG_{GDP} complexes can become spontaneously activated in the absence of an agonist, thereby permitting GDP release and GTPγS binding. The inverse agonist rimonabant stabilized all three receptor–Gαi complexes as long as GTP analogs were not present. GTPγS promoted dissociation of G proteins from receptors, and this response was partially reduced by rimonabant [14]. For other GPCRs

that do not exist as stable ternary complexes (agonist–receptor–G protein (ARG)) in detergent solution (e.g., receptors for somatostatin, δ-opioid, and β_2-adrenergic agonists), the receptor-G protein (RG) association could be promoted by agonists as long as GTP or GTPγS were not present [19–22]. This difference in the ability of the receptor to be coupled to the G protein in the absence of an agonist might explain why the CB1 receptor can exhibit constitutive activity.

2.2 Inverse Agonists and Allosteric Regulators of CB1 Responses

The mechanism by which the receptor responds to rimonabant as an inverse agonist has been explored using analogs and mutated CB1 receptors [23]. In those studies, inverse agonist activity was determined by the ability of rimonabant to increase the Ca^{2+} current in superior cervical ganglia neurons expressing exogenous CB1 receptors. Rimonabant's inverse agonist activity was not observed in mutant CB1 receptors in which the transmembrane helix 3 Lys residue was mutated to Ala, even though competitive antagonism of the WIN55212-2 response was not affected [9, 23]. An analog of rimonabant that lacks the hydrogen-bonding amide oxygen was not able to promote inverse agonist activity, but was able to compete with WIN55212-2. These studies support the model of an interaction of the rimonabant with the helix 3 Lys via the amide oxygen to produce inverse agonist activity, but not for competition with agonist [23]. One explanation is that the CB1 receptor may have two different binding conformations for rimonabant, one of which, R^o, is associated with inverse agonist activity, whereas an R' conformation may exist as a state in which agonists and antagonists can compete with each other for binding. An alternative is that rimonabant may bind at two different binding loci within the receptor-binding pocket, one of which confers inverse agonist activity. The two-loci binding model is supported by biochemical studies of Sim-Selley and colleagues [10].

The notion that a ligand might bind to two different binding loci within the binding pocket has gained support from the finding of a series of allosteric regulators of the CB1 signal transduction response (Org27569, Org27759, and Org29657) [3]. Radioligand-binding studies demonstrated that the allosteric ligands could increase the binding of [^{3}H]-CP55940 by decreasing the rate of dissociation of this agonist. In contrast, the allosteric ligands could compete for binding of [^{3}H]-rimonabant, but the competition did not extend to the entire population of binding sites for [^{3}H]-rimonabant. This finding might suggest that these allosteric ligands were binding to a site that was distinct from that of the agonist CP55940, and distinct from a certain subset of rimonabant sites. The finding of competitive binding might suggest that there is a separate and distinct binding site for rimonabant that is shared in common with the allosteric regulators. Although the allosteric regulators alone had no agonist or inverse agonist effect on signal transduction leading to regulation of electrically evoked

contractions in the mouse vas deferens model or agonist-stimulated response in a reporter gene expression system, these regulators could antagonize the agonist-stimulated response in a noncompetitive manner [3]. These findings were consistent with an abrogation of agonist-mediated G-protein activation in the presence of the allosteric regulators [3]. The possibility that rimonabant and other inverse agonists may utilize this site in addition to a site that competes directly for agonist binding could explain the complex regulation of signal transduction observed previously by other investigators [8, 24, 25].

Very intriguing research findings have led to the proposal that CB1 receptors bound to rimonabant can sequester Gi/o proteins to alter signal transduction in the cell involving other receptors. Bouaboula and colleagues [7] observed that rimonabant could block signal transduction pathways stimulated by receptors that might share G proteins with the CB1 receptor. The strongest support for the notion of G-protein sequestration comes from studies of Lewis and colleagues, who investigated the regulation of Ca^{2+} channels by CB1 receptors [9, 11]. When recombinant CB1 receptors were expressed in superior cervical ganglia neurons, the responses to norepinephrine or somatostatin were attenuated, suggesting that the endogenous α_2-adrenergic and somatostatin receptors shared a pool of Gi/o proteins with the exogenous CB1 receptors [11]. Further evidence for shared pools of Gi proteins comes from studies of human embryonic kidney (HEK293) cells stably expressing D_2-dopamine receptors and transiently expressing CB1 receptors [26]. In those studies, D_2-dopamine receptors sequestered Gi proteins, preventing their coupling to the CB1 receptors, relegating the CB1 receptors to couple to Gs proteins instead. The net result was that the agonist CP55940 stimulated CB1 receptors to increase cyclic 3',5' -adenosine monophosphate (cyclic AMP) production rather than to decrease cyclic AMP production as observed in the absence of exogenous D_2-dopamine receptors. Overexpression of Gαi1 reversed this pattern of response, permitting the inhibition of cyclic AMP accumulation by CP55940-stimulated CB1 receptors [26]. Furthermore, CB1 receptor–Gi coupling was observed if the D_2-dopamine receptors were desensitized by persistent stimulation with quinpirole such that these receptors could no longer couple to Gi [26].

There is evidence to argue against other GPCRs sharing a common pool of G proteins with CB1 receptors. For example, cerebellar granule cells, which endogenously express cannabinoid receptors and γ-aminobutyric acid ($GABA_B$) receptors, do not share a common G-protein pool as determined by the additivity of baclofen-stimulated and cannabinoid-stimulated low Km GTPase activity [27, 28]. However, cotreatment of cultured granule cells with baclofen and cannabinoid agonists failed to produce an additive response for inhibition of intracellular cyclic AMP, suggesting that these receptors may share a common pool of adenylyl cyclase. The additivity of opioid- and cannabinoid-stimulated GTPγS binding to G proteins in N18TG2 membranes suggested that these receptors were coupled to separate pools of G proteins [29]. This was true even with limiting amounts of G proteins after depletion of the Gi pool with pertussis toxin. Chronic exposure of N18TG2 cells to the potent

opioid agonist “etorphine” attenuated, rather than augmented, the cannabinoid activation of G proteins [29]. Chronic treatment with the potent cannabinoid agonist desacetyllevonantradol resulted in desensitization to cannabinoid, but not the opioid agonists. These findings suggest that the uncoupling of the opioid receptors failed to increase the pool of G proteins available for coupling to the cannabinoid receptors, and vice versa. In contrast, cotransfection of COS-7 cells with CB1 cannabinoid receptors and either μ- or δ-opioid receptors resulted in the combined effect of agonist stimulation of the two receptor types as being the same as either one alone [30]. Such findings suggest the activation of a common G-protein pool by the two receptor types. The difference between these results and those in N18TG2 cells is that the latter endogenously coexpress CB1 and δ-opioid receptors, and the membrane localization, protein stoichiometry, and protein complex organization of the receptors and G proteins might be different between endogenously expressed and transiently exogenously expressed proteins.

3 Agonist-Directed Stimulation of Selective G Proteins

3.1 Differential G-Protein Activation by Agonists

GPCRs can couple differentially to different Gα subtypes depending upon the agonist and the cellular environment [31]. The term *agonist trafficking* has been used to describe the agonist-promoted coupling of receptor to a selective G protein leading to differential signal transduction in a cell. The concept that a receptor can be regulated by different agonists to signal selectively through different G proteins implies the possibility for multiple ternary complex equilibrium models having activated receptor states that may be distinct for different G proteins [32–35]. For other GPCRs, it has been shown that coupling to multiple G proteins can occur within the Gi/o subfamily [36–38].

For the CB1 receptor, investigations of G-protein activation have found differences in agonist efficacy to promote the exchange of a GTP analog for GDP on the Gα subunit. Glass and Northup demonstrated agonist-selective activation of G proteins in a recombinant system [39]. These researchers examined the activation of [^{35}S]GTPγS binding to purified Gαi (all subtypes) and Gαo proteins reconstituted with recombinant CB1 receptors in Sf9 cell membranes. HU210 maximally stimulated both Gαi and Gαo, whereas Δ^9-THC poorly stimulated either Gαi or Gαo. WIN55212 and anandamide maximally or near--maximally stimulated Gαi, but only partially (70%) stimulated Gαo. Rimonabant inhibited [^{35}S]GTPγS binding to both Gαi and Gαo. Prather and colleagues reported differences in the potency for WIN55212-2 to stimulate [^{32}P]azidoanilido-GTP binding to different G proteins in rat cerebellum membranes [40]. In those studies, WIN55212-2 activated binding to Gαi1 and Gαo3 with an ED_{50} of 100 nM, but activated binding to Gαo2 with an ED_{50} of 3.7 μM.

3.2 Receptor-G-Protein Equilibrium: Influence of Agonists versus Inverse Agonists

Howlett and Mukhopadhyay [14] hypothesized that structurally distinct ligands would exhibit differential ability to regulate CB1 receptor interactions. This hypothesis was tested using detergent-solubilized receptor–Gα complexes, with the dissociation of the ternary complex as the measure of G-protein activation. In response to agonist-induced dissociation of GDP from the inactive Gα, an empty AR*G_ complex would exist only transiently in intact cells because GTP would fill the guanine nucleotide-binding site resulting in AR^*G_{GTP}. In the absence of GTP, this complex could revert to ARG and dissociate to AR + G. In studies of these complexes in detergent solution [14], the aminoalkylindole WIN55212-2, the cannabinoid desacetyllevonantradol, and the eicosanoid (R)-methanandamide promoted an equilibrium mixture of receptor bound to G proteins (RGαi) and receptor in a free form (AR or R). The interesting finding from these studies was that the three agonists promoted dissociation of the complex differently, depending upon the Gαi subtype. The highly efficacious agonist WIN55212-2 promoted dissociation of the CB1 receptor from all three Gi subtypes, in the presence or absence of GTPγS [14]. Desacetyllevonantradol promoted dissociation of receptors from Gαi1 and Gαi2 only, and (R)-methanandamide promoted dissociation of receptors from Gαi3 only. This finding suggests that an isomerization of selective G proteins to AR*G was induced differentially by these ligands, qualifying them as agonists for these G-protein subtypes.

In the presence of GTPγS, the agonist would promote an $AR^*G\alpha_{GTP\gamma S}$ ternary complex, which would dissociate and would not be likely to reassociate. GTPγS converted the majority of CB1 receptors in the high-affinity state for WIN55212-2 (ARG_{GDP} or empty AR*G_) to the low-affinity state (AR) [16]. However, a majority of the CB1 receptors remained in the high-affinity state for desacetyllevonantradol [16]. This is consistent with the finding that GTPγS was able to promote dissociation of all three Gi subtypes from the CB1 receptor in the presence of WIN55212-2, but that GTPγS failed to evoke dissociation of Gi3 in the presence of desacetyllevonantradol [14].

The inverse agonist rimonabant did not allow dissociation of the G protein from the CB1 receptor, and this result was also observed for desacetyllevonantradol with Gi3, and (R)-methanandamide with Gi1 and Gi2 [14]. This finding would be consistent with the conversion of the RG_{GDP} complex to a sustainable IR^oG_{GDP} complex. The property of these ligands to behave as inverse agonists for these G-protein subtypes would also explain the ability of desacetyllevonantradol or (R)-methanandamide to preclude the ability of GTPγS to drive forward the dissociation of Gi3 (desacetyllevonantradol), or Gi1 and Gi2 ((R)-methanandamide). Judging by the ability to disrupt the GTPγS-driven dissociation of $G\alpha i_{GTP\gamma S}$, the inverse agonist efficacy to promote the isomerization to IR^oG_{GDP} was greater for desacetyllevonantradol at Gi3, and for

(R)-methanandamide at Gi1 and Gi2, than it was for rimonabant at any of the Gi subtypes. These results provide evidence for the differential behavior of these ligands as agonists or inverse agonists depending upon the Gi subtype.

If one considers the dissociation of the AR*G_ complex to be the hallmark of an agonist and stabilization of the (I)R°G complex as the hallmark of an inverse agonist, then we would have to accept the notion that the CB1 receptor ligands are able to behave as either agonists or inverse agonists depending on the subtype of the G protein. The observation that WIN55212-2 appears to behave as a full agonist in nearly every signal transduction assay studied (see [4] for review), is consistent with its ability to dissociate all three RGαi subtypes. In contrast, anandamide (and by inference, its metabolically stable analog (R)-methanandamide) has been reported to behave as a weak partial agonist (see [41] for review and original references]. This ligand was not able to dissociate receptor complexes with Gαi1 and Gαi2. This observation might be considered in pharmaceutical drug design. Thus, it is likely that the endogenous ligand anandamide can activate only a limited number of signal transduction pathways in which Gαi3 is the dominant G protein in promoting the response. In contrast, drugs based upon the WIN55212-2 structure could be predicted to activate a much broader range of signal transduction pathways because of its agonist ability for a multiplicity of Gi subtypes.

3.3 CB1 Receptor Coupling to Gs

Data from several studies have drawn attention to the ability of the CB1 receptor to couple to Gs under conditions of pertussis toxin-treatment. When pertussis toxin prevented the receptor's interaction with Gi/o proteins in primary neuronal cultures and CHO cells expressing recombinant CB1 receptors, cyclic AMP was increased in response to cannabinoid agonists [42, 43]. This response could be blocked by CB1 cannabinoid receptor antagonists, demonstrating that only the CB1 receptor need to be involved. In CHO cells expressing exogenous CB1 receptors, pretreatment with pertussis toxin resulted in stimulation of cyclic AMP accumulation, presumably by Gs [44]. WIN55212-2 increased cyclic AMP levels by 100% above the forskolin-stimulated level, but HU210 and CP55940 increased levels by only 50%, and Δ^9-THC and anandamide by about 35%. The potency order to stimulate cyclic AMP production was the same as the inhibition of cyclic AMP production in the absence of pertussis toxin pretreatment, and the stimulation could be blocked by rimonabant, indicating that the CB1 receptor regulated both responses.

The studies of cells treated with pertussis toxin to eliminate CB1–Gi interactions would imply that the coupling to Gs must require different agonist-induced conformational changes than required for Gi. After pertussis toxin treatment, a mutant CB1 receptor that contains a Gs-coupling sequence (Ala-Leu), rather than Leu-Ala found in the CB1 receptor, was able to couple

to Gs [45, 46], suggesting that the mutant CB1 receptor could associate with Gs as might be predicted from sequence homologies with Gs-coupled receptors.

An interesting phenomenon has linked stimulation of cyclic AMP production with the combination of cannabinoid and dopamine receptors. Using striatal cell culture as a model, Glass and Felder found that stimulation of dopaminergic and cannabinergic receptors together resulted in an increase in cyclic AMP, whereas stimulation of either one alone resulted in the typical Gi-mediated decrease in cyclic AMP [42]. In globus pallidus slice preparations, which would comprise intact axonal terminals that possess both cannabinoid and dopaminergic receptors, WIN55212-2 produced an increase in basal cyclic AMP accumulation [47]. In order to investigate this phenomenon at the cellular level, Jarrahian and colleagues transiently expressed CB1 receptors in HEK293 cells with or without stably expressed D_2-dopamine receptors [26]. In cells expressing the CB1 receptors alone, CP55940-inhibited forskolin-stimulated cyclic AMP production was observed. In contrast, in the CB1 and D_2 coexpression model, CP55940, a CB1 agonist, stimulated cyclic AMP production [26]. CP55940 stimulation increased the net cyclic AMP accumulation in studies of dopaminergic inhibition of forskolin-stimulated cyclic AMP production. These studies are consistent with a stimulation of Gs by the agonist-stimulated CB1 receptor. The CB1 receptor–Gs stimulation was not as great as the forskolin-stimulated response, and required 0.1–10 μM CP55940 compared with 10–1000 nM CP55940 for inhibition of cyclic AMP [26]. These findings were reexamined by Kearn and colleagues, who stably co-expressed Flag-tagged D_2 receptors with hemagglutinin-tagged CB1 receptors in HEK293 cells, and found that the inhibition of forskolin-activated cyclic AMP production could be reversed by increasing concentrations of the combined agonists [48]. These researchers further showed that in the presence of the combined agonists (but not either one alone), the D_2 dopamine and CB1 cannabinoid receptors formed coimmunoprecipitable complexes which were stable in CHAPS detergent conditions for immunoprecipitation, but not in sodium dodecylsulfate (SDS) detergent for polyacrylamide gel electrophoresis [48]. These findings lead to the novel hypothesis that heterodimerization of these two GPCRs in their activated states leads to a modification of the G-protein selectivity.

4 Coupling of the CB1 Receptor to G Proteins

4.1 Intracellular Domains Critical to G-Protein Activation

GPCR residues that interact with the coupled G protein are mainly from IL2, IL3, and helix 8 in IL4 [49, 50]. See Fig. 2 for CB1 sequence structure. It has been suggested that GPCR intracellular loops directly interact with G proteins [49, 51, 52]. These intracellular loops are important for determining specific G-protein coupling [53] among 18 different subtypes of G proteins

[54]. In particular, (IL3), located in the central region of the 7-transmembrane (TM) bundle, appears to play an important role in transferring the molecular signal to the coupled G proteins, resulting in G-protein activation. Mutation of rhodopsin IL3 and its neighboring residues at the C-terminal end of TM helix 5 (TM5) and the N-terminal of TM6 showed significant reduction in Gt activation [55, 56], suggesting the important role of IL3 in G-protein coupling and activation.

In the β_2-adrenergic receptor, the C terminus appears to be essential for interaction of the G protein with the receptor, demonstrated by normal ligand binding but reduced signaling in truncated mutant receptors [57]. Both the IL3 and the C-terminal domain of the β_2-adrenergic receptors were shown through the use of β_1/β_2-adrenergic receptor chimeras to be important for spontaneous activity, while not being essential for ligand binding [51]. The crystal structure of rhodopsin shows a short helical segment in the intracellular tail extending from TM7. This helix was shown to move upon photoactivation of the receptor,

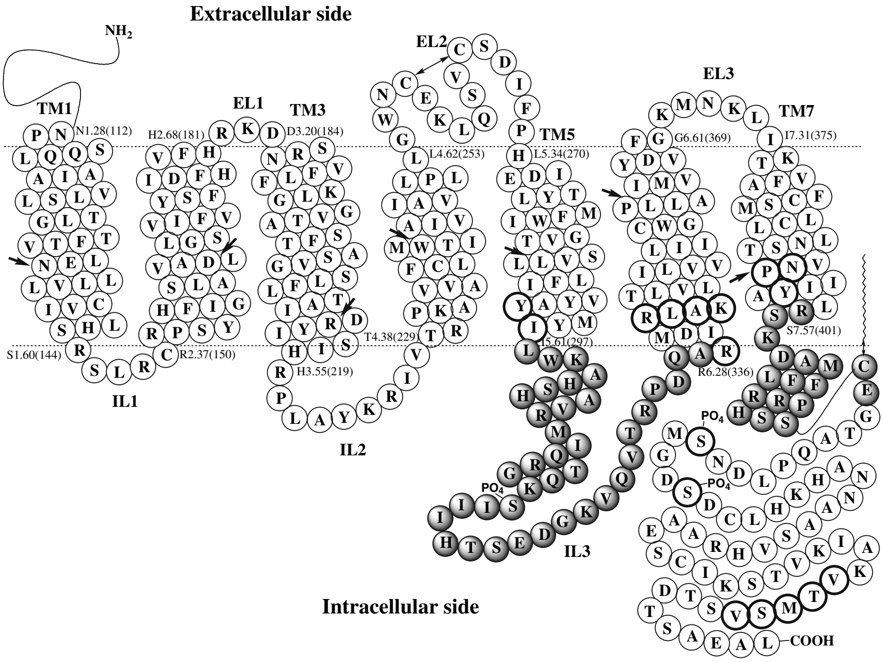

Fig. 2 The human CB1 receptor in two-dimensional representation. Three extracellular (EL1, EL2, and EL3) and intracellular (IL1, IL2, and IL3) regions are presented. Residues are numbered using the helix number to the left of the decimal, and the most conserved residue in each helix designated as 50 to the right of the decimal, as indicated by an arrow (described for the CB1 receptor by Bramblett and colleagues [78]). The palmitoylation of a C-terminal Cys residue is depicted as a membrane attachment locus. Shaded regions show loops IL3 and the juxtamembrane region of IL4 (helix 8). Specific amino acid residues discussed in the text are depicted in bold type

detected through cys cross-linking studies [58] and nitroxide spin label studies [59]. This region was shown to be involved in the coupling of rhodopsin to Gt [49].

The juxtamembrane C-terminal, extending from TM7 to one or more palmitoylated Cys residues, defines the IL4 domain. The IL4 of rhodopsin possesses two palmitoylation sites, C322 and C323 [60]. In CB1, the juxtamembrane region consists of residues R400-E416 in the human receptor, R401-E417 in the rat receptor (and specific residue numbers are for the human receptor unless otherwise indicated by an "r"). C415 is a likely site for palmitoylation of the cannabinoid receptor, providing an anchor for the IL4 into the cell membrane. Studies of peptide CB1 rR401-E417 show that this peptide is effective at G-protein activation when C415 is mutated to a ser, suggesting that the peptide need not be anchored into the membrane to be effective [61]. The hCB1 receptor as a fusion protein to Gαi was synthesized with C415 mutated to Ala [62]. The C415A receptor exhibited similar functionality to wild-type receptors with respect to ligand binding and stimulation of GTPγS binding to G proteins. However, the authors caution that signaling changes may be obscured due to tethering of the G protein to the CB1 receptor. Mutation of C313 and C320 in CB2 receptors did not change ligand binding, receptor expression, or desensitization, but attenuated adenylyl cyclase inhibition [63].

Chimeric receptors wherein the C terminus of CB1 was replaced with the C terminus of CB2 exhibited a twofold increase in affinity for rimonabant [64]. A series of truncation mutants were developed to examine the action of phosphorylation sites on the C-terminal domain. Truncation at rA439 and rT460 resulted in desensitization to inwardly rectifying potassium channels similar to that of the wild-type receptor upon exposure to WIN-55212-2 [65]. Truncation at T418, however, produced only slight desensitization, implying that an essential phosphorylation site for this process can be found in the region between residues 418 and 439. The data indicate that mutations rS426A and rS430A each individually attenuated desensitization. Residues rV460 to rV464, but not rS426 or rS430, were shown to be necessary for internalization of the receptors [65, 66].

Several effects were studied from deletion mutations of the C-terminal tail [67, 68]. Deletion of the C-terminal tail abolished the ability of the receptor to inhibit the Ca^{2+} current. The juxtamembrane domain was shown to be critical for G-protein coupling, but the distal region from residue 418–472 was shown to modulate the ability of the receptor to couple to G proteins. These changes were not caused by deviations of expression or transport to the membrane surface.

4.2 Sequence, Environment, and Structure

The IL3 regions of the rhodopsin family of GPCRs show a high degree of sequence and size heterogeneity. In particular, the proximal N-terminal residues at the end of TM5 and the distal C-terminal residues at the beginning of TM6 were predicted to be dynamically modified to form different secondary

structures that would influence the folding status of IL3 [69]. For example, a recent X-ray structure of rhodopsin [70] reported the IL3 conformation being a helix-turn-helix, which is consistent with the possible secondary structure consisting of H-bonded turns by a spin-label study [55]. Similarly, a nuclear magnetic resonance spectroscopy (NMR) study on the IL3 of the rat AT_{1A} angiotensin II receptor showed that the proximal N-terminal (residues 213–231) and the distal C terminal (residues 236–241) adopt somewhat stable α-helical structures [71].

Compared with the rhodopsin IL3, which contains 18 amino acids (V227-Q244), the CB1 receptor IL3 is longer and contains 38 amino acids (L298-A335). From an NMR study on the IL3 peptide of the CB1 receptor in SDS micelles [72], a possible conformation of IL3 has been reported. The IL3 structure of the CB1 receptor showed fascinating features including several α-helical structures (i.e., W299-R307 and I309-S316), the C-terminal fragment I317-Q334 with an extended conformation, and an Ile-Ile-Ile triad anchoring to the hydrophobic phase of the cell membrane. It appears that the N-terminal fragment I309-S316 forming an amphipathic α-helix would interact with the G protein [72]. It is likely that different GPCRs would make use of sequence and structural variance for generating unique molecular signals from ligand binding and for delivering signals to selective G-protein subtypes.

The X-ray structure of rhodopsin shows a helical structure at the beginning of the IL4 domain [73]. Examinations by NMR of the IL4 of rhodopsin in different media show a random coil structure in aqueous media and helical structure in detergent micelles [60]. Several other GPCRs, including the β_2-adrenergic receptor [74], AT_{1A} receptor [75], and the turkey adrenergic receptor [76], were shown to possess similar environmentally dependent structural features at the N-terminal side of IL4. Structural homology persists even in the absence of primary sequence similarity, and evidence suggests that environment-induced conformational changes in this region are involved in G-protein signaling. Activation of G proteins by the juxtamembrane region is implicated for rhodopsin [50] and β-adrenergic receptors [77].

A calculated structure of CB1 defined an intracellular helical segment at the end of TM7 and separated from it by Arg and Lys residues, which are presumably located at the negatively charged phospholipid headgroups at the membrane interface [78]. The CB1 rR401-E417 juxtamembrane region was shown to have helical character in SDS and dipalmitoylphosphatidylglycerol (DPPG) micelles by circular dichroism spectroscopy (CD), but no structure in sodium phosphate buffer or methanol [61]. Studies of a peptide restricted in its conformations suggest that α-helical structure is not a requirement for the CB1 peptide to stimulate Gi functions in membrane preparations [61]. CD spectroscopy in dodecylphosphocholine (DPC) micelles, a zwitterionic membrane-mimetic, indicated that the peptide had 65% helical character [79]. Removal of R401 resulted in a truncated peptide that had increased helical character in trifluoroethanol (TFE), although this change resulted in loss of affinity for G proteins and loss of activity for inhibition of adenylyl cyclase [61].

NMR was performed on peptide fragment rCB1 I397-G418 in DPC micelles [79], and showed two helical regions located between residues rI397-L400 and rS402-F413, with a bend occurring at rR401. A second independent study of the same peptide in DPC micelles supported these results [80]. The rR401A modification resulted in the formation of a single helix that continued through rF413 [79]. Spectroscopically obtained distance constraints were determined using nuclear Overhauser enhancement (NOE) spectroscopy and suggested that the juxtamembrane helix is aligned parallel to the micelle surface [79]. Xie and Chen envisioned a salt bridge between rD404 and rH407 [80]. A study of the peptide in SDS, a negatively charged membrane mimetic, also showed helical character, but a lack of NOEs between the α proton of residue i and amide proton of $i + 4$ suggests that the peptide exists primarily in a 3_{10}-helix with the helical structure in SDS between rL405 and rF413 [81].

The IL3 of GPCRs, directly interacting with the coupled G proteins, is important in determining specific coupling and in transferring the molecular signal resulting in G-protein activation. In GPCRs, juxtamembrane regions of the IL3 loop have been found to participate in G-protein coupling/activation [82]. For example, it was revealed from a gain-of-function mutation study by Natochin and colleagues [50] that the IL3 residues (T229/V230 and S240/T242/T243/Q244) proximal to TM5 and TM6 were more important than those residues in the middle of IL3 (K231 through E239) for transducin (Gt) interaction and activation. Furthermore, residues at the positively charged C-terminal side [83] and nonpolar N-terminal side [53, 56, 84–89] appear to be important for G-protein activation. In mutation studies of the m5 muscarinic receptor, Burstein and colleagues identified a combination of charged and hydrophobic residues at the juxtamembrane regions of IL3 that were critical for G-protein coupling, proposing that these residues formed a G-protein coupling pocket [90, 91]. These data show that it is not easy to clearly state that one particular class of residues in a certain region is important for G-protein activation.

It has been suggested that ligand-specific receptor conformational changes at the intracellular side interacting with G proteins would be critical in coupling to ligand-specific G-protein activation. The CB1 receptor functional interaction with G proteins was studied in vitro using peptides and site-directed antibodies [92]. It was found that peptide fragments from the N-terminal side of IL3 and the C-terminal juxtamembrane IL4 promoted autonomous Gi activation and inhibition of adenylyl cyclase activity, which suggests that these peptides represent two domains on the receptor that interact with and contribute to the activation of Gi [18].

The G-protein C-terminal residues (340–350), as a structured form of the Gα subunit [93–95], might directly interact with the cytoplasmic side of GPCRs [96]. It has been suggested from cross-linking studies that the N- and C-terminal residues of Gα should be located close to the region homologous to the Y136-V139 and E247-E249 of rhodopsin [52, 97, 98]. Thus, it is likely that interactions with G proteins can provide charge–charge interactions at the GPCR intracellular surface that would support initiation or stabilization of helical structure

during the G-protein activation cycle. The negatively charged amino acids of the Gα C-terminal may interact with positively charged amino acids within the receptor intracellular helices. For rhodopsin and other GPCRs, this may involve IL3 and IL4 helices [99, 100]. Hydrophobic residues could also be important to provide an interaction face to the coupled G protein.

Spacing of cationic residues along the primary sequence of the IL4 peptide results in an amphipathic helix. The cationic, multibasic sequence formed by three Arg residues and a Lys residue appears to be important for determining the efficacy of the peptide to inhibit adenylyl cyclase, since acylation of rK403 resulted in a peptide with apparent affinity for the G protein, but decreased efficacy [61]. One structure reported by Xie and Chen positions these cationic residues pointing down into the cellular space, and presumably toward the G protein [80]. This orientation is based largely on NOE data that show close through-space interactions between rA399 and rL405. These data are also in agreement with the published X-ray structure of rhodopsin [73]. A second orientation, inferred from ^{31}P-^{1}H NMR NOE data showing close through-space interactions between phosphate head groups and rR406 and rR410 in DPC micelles, envisions the positively charged residues pointing toward the negatively charged head groups of the phospholipids, with other noncationic polar residues extending into the cytoplasmic space [79]. It was proposed that activation of the receptor could bring about conformational changes that would release this anchoring into the membrane surface, thereby exposing the peptide to interaction with the G protein.

The negative character of SDS creates micelles that suggest the anionic surface of the membrane bilayer or possibly a patch of negative residues on the G-protein surface. The calculated peptide structure in SDS did not rule out either possible orientation of the peptide proposed by the studies taken in DPC [81]. With the NMR data suggesting that the peptide may possess more 3_{10}-helical character under a negatively charged environment, it was proposed that a conformational change from α- to 3_{10}-helix may be the mechanism for G-protein signaling observed upon receptor activation. The change in peptide length between these two structures is consistent with the changes in IL4 observed upon activation of rhodopsin [59]. Calculations for insertion of an amphipathic helix into a membrane bilayer have shown that an amphipathic helix of this nature could embed itself deeply enough that the cationic residues can point toward the cytosol and still be interacting with the lipid head groups [101].

The juxtamembrane C-terminal region of the CB2 receptor was predicted to possess a helical region between G304 and L314 [102]. Peptide CB2 I298-K319 was evaluated by CD for secondary structure in two solvents [103]. In water, 12% helical character was exhibited as compared to 85% in DPC micelles. NMR experiments in water indicated no secondary structure. NOE experiments in DPC indicate the presence of two helical portions, from I298-L301 and S303-K310, with a bend at the connecting R302. A Pro is lacking in the CB2 peptide to terminate the helical structure as in the CB1 peptide. NOE

through-space interactions between A300 and I306 support a turn that orients the peptide approximately parallel to the cell membrane. NMR studies in dimethylsulfoxide (DMSO) give similar results with a helical structure present throughout much of the peptide [103]. Computational prediction of the peptide structure using distance-restrained molecular dynamics points to a salt bridge between R302 and E305 that is not present when E305 was replaced by Gln [80]. C313 is a probable site of palmitoylation, suggesting that its side chain is oriented toward the membrane. The CB2 peptide structure has several cationic hydrophilic residues aligned on the same side of the helix, affording an amphipathic character with the positive charge oriented toward the cytoplasmic space.

There are several explanations for the conformational changes seen in different environments by the juxtamembrane regions. Studies with amphipathic helices have shown binding that is frequently parallel to the surface of phospholipid bilayers, with the hydrophobic residues extending into the core and the positively charged residues facing toward the cytoplasm or embedded in the phospholipid head groups [104]. Solvent-dependent conformational changes may be indicative of the changes that ensue when the region is bound to the G protein and is thus engaged in activation of the signaling pathway. The data are inconclusive regarding whether the peptide exists in a helical conformation or a random coil configuration when the peptide is interacting with a G protein. The induced helical structure may also serve as a regulatory mechanism by anchoring the peptide into the membrane, thus concealing the binding regions from the G protein.

4.3 G-Protein Coupling Specificity

G-protein subtype preference has been demonstrated for serotonin $5HT_{1A}$ [105], D_2-dopamine receptors [106], and A_1 adenosine receptors [107]. The IL3 of many GPCRs contains residues or motifs that recruit specific subtypes of G proteins. A mutational study by Yamashita and co-workers [108] showed that by replacing the IL3 loop of rhodopsin with that of several representative GPCRs that are preferentially coupled to Go, the mutated receptors accordingly activated Go, but not Gt. This study suggested that the IL3 of rhodopsin contains residues specific to Gt coupling. A mutation study by Senogles and colleagues [109] showed that the D_{2S} (short) dopamine receptor coupled preferentially to Gαi2, whereas the R233G and A234T mutants coupled preferentially to Gαi3 and Gαi1, respectively. This finding suggests that these specific middle residues of IL3 in the D_2 receptor alter G-protein specificity. The authors proposed that these point mutations would alter the presumed helical structure of the IL3 loop around the mutated region and its coupling interaction with G proteins. A similar conclusion was drawn for the AT_1 receptor [110]. Using AT_1/AT_2 chimeric receptors, these researchers observed that AT_1 receptor activity was not affected by replacement of IL2 and IL4 between AT_1 and AT_2 receptors. However, replacement of AT_1 IL3 by AT_2 IL3 abolished activity, and replacement of AT_2 IL3 by AT_1 IL3 conferred the AT_1 activity.

A detailed experiment showed that only the N-terminal and C-terminal residues of IL3 proximal to TM5 and TM6, particularly W219-A225, were critical for G-protein coupling. A similar chimeric study by Conchon and co-workers [111] restated the importance of the C-terminal residues of IL3 of the AT_1 receptor in selective G-protein coupling.

It was shown that cannabinoid receptors couple to various subtypes of G proteins, including Gi [112], Gs [42], and Go [39]. Glass and Northup [39] observed that CB2 receptors interacted well with Gi, but not with Go. Howlett and colleagues [18, 91, 113] detailed through in vitro studies that IL3 of the CB1 receptor interacted with Gi1 and Gi2, whereas H8 interacted with Gi3 and Go. To characterize a role of the C-terminal region of IL3 of the CB1 receptor in G-protein selectivity, Kendall and colleagues [45] studied a double mutation L341A/A342L, which resulted in the signature motif for Gs coupling in the β2-adrenergic receptor (Ala-Leu-Lys-Thr) [114]. This change caused the mutated receptor to be partially constitutively active selectively for Gs, not for Gi. Recently, the NMR structures of these C-terminal IL3 residues of the CB1 receptors in complex with Gαi1 showed a helical peptide structure, whereas the peptide with the Ala-Leu motif had a single turn structure [46], suggesting the requirement of an α-helical conformation of these C-terminal IL3 residues for efficient Gαi1 binding. It is possible that these C-terminal IL3 residues might regulate the geometrical arrangement of the distal portion of IL3, which contains several charged residues (K326, R331, D333, R336, D338, R340, etc.) that modulate G-protein coupling and activation.

The IL4 peptide CB1 rR401-E417 was shown to autonomously activate Gi proteins to inhibit adenylyl cyclase in a dose-dependent, pertussis toxin-sensitive manner [91]. The peptide selectively competed for subtypes Go and Gi3 but not Gi1 or Gi2. This suggests that the peptide occupies a high-affinity site available on select G-protein subtypes [18, 114]. The CB1 rR401-E417 peptide-induced inhibition of forskolin-stimulated adenylyl cyclase activity was unaffected by rimonabant, the CB1 antagonist, indicating that the peptide-mediated activation of Gi did not require cannabinoid receptors [113]. It is believed from these results that the peptide acts as a mimic for the receptor domain that activates these G-protein subtypes [91]. Inhibition of adenylyl cyclase by CB1 rR401-E417 was unaffected by the addition of CB2 L302-K320, showing that the juxtamembrane region of CB2 does not act competitively against the CB1 peptide for the Go and Gi3 binding sites [113].

4.4 Functional Motifs for G-Protein Coupling and Activation

Several distinct sequence motifs within the IL3 or IL4 regions were proposed to be important for G-protein coupling. These motifs would be sequence and structurally specific in order to generate unique molecular signals from ligand binding and for delivering signals selectively to different G-protein subtypes.

4.4.1 YXXIXXL/A Motif

It was revealed from a comparison of the N-terminal residues of IL3 of many GPCRs that a YXXIXXL/A motif, in which X represents any residue, is highly conserved and an extension of the helical structure [115]. With a basic residue followed by a nonpolar residue Leu (or Ala), the motif would form an amphiphilic helical structure for G-protein coupling and activation. The terminal L/A residue of this motif was proposed to form a hydrophobic cluster with neighboring hydrophobic residues in TM5 and TM6 [85], which might be critical for G-protein activation. For example, in the parathyroid hormone (PTH) receptor, nonpolar residues (V378 and L379) were important for the Gq-phospholipase C signaling pathway, whereas polar (T381) and positively charged (K382) residues were important for the Gs-adenylyl cyclase signaling pathway [86].

It was reported that the positively charged residues at the IL3 N-terminal of the AT_{1a} were not important for G-protein activation [83]. A mutation study showed that a nonpolar residue L237 at the N-terminal region of IL3 of GnRH was important for G-protein activation [85]. Similarly, the corresponding residue of the AT_{1a} receptor was important for G-protein function [115]. The CB1 receptor contains the corresponding **Y**MY**I**LWK**A** (294–301) sequence at the C-terminal end of TM5, and this sequence was predicted to form an amphipathic α-helical structure according to an NMR study [72].

4.4.2 BBXB or BBXXB Motif

The α2-adrenergic receptor possesses a highly charged C-terminal domain of the IL3, which has been shown to interact with and activate G proteins. Okamoto and Nishimoto proposed that a motif of BBXXB or BBXB, where B represents a basic residue and X represents a nonbasic residue, on the C-terminal domains of intracellular loops was required for Gi activation [116]. Mutation studies of adrenergic receptors have indicated that three Arg residues distal to the membrane (BXBXB) are important for Gs activation, whereas the BXBB motif at the C-terminal domain of IL3 is important for Gi activation [117]. Mutation studies revealed that the basic residues of the conserved BBXXB motif at the distal IL3 were critical in the μ-opioid receptor (especially R280) for Gi-protein activation [118], but not in the M_2-muscarinic receptor for Gi/o-protein activation [119]. It was reported that the N-terminal sequence of AT_1 receptor IL3 W**K**AL**KK**A (219–225) was the BXXBB moiety critical for Gq coupling. There are three Arg and one Lys nonconsecutive residues within the sequence of the CB1 C-terminal domain, **R**MDI**R**LA**K** (336–343) posing the possibility for a cationic patch. These studies would suggest that heterogeneity of IL3 is functionally significant [119].

4.4.3 TM7 and IL4 NPXXY(X)$_{5,6}$F

Rhodopsin contains an NPXXY(X)$_{5,6}$F motif that employs aromatic stacking to link Y306 in TM7 to F313 in H8. This interaction may play a role in the

photoactivated structural rearrangement [120]. In the human β_1-adrenergic receptor, mutation of the Phe residue to His prevented the proper biosynthesis of the receptor [121].

The NPXXY$(X)_{5,6}$F motif is found in many mammalian class A GPCRs, but is not conserved in CB1, in which Phe is replaced by a Leu residue. Abood, Reggio, and colleagues hypothesized that the differences in this motif in the CB1 receptor could explain certain unique properties of the CB1 receptor signaling [122]. Two mutated CB1 receptors were synthesized, L(404)F and L(404)I, and tested for ligand recognition, receptor activation, and changes in G-protein coupling [122]. The three receptors showed similar receptor expression levels and similar ligand-binding affinity for CP55940 and rimonabant. The two mutant receptors showed a significant reduction of stimulation of GTPγS binding to G proteins in response to CP55940 and WIN5212-2. Internalization of the receptors occurred faster for the two mutant receptors than for the wild type, suggesting that the mutants may exhibit altered response to agonists. The mutants showed coupling with Gαi1 and Gαi2, but not with Gαi3, consistent with the notion that the IL4 is involved in the interaction with Gαi3. It is theorized that the mutation of the Leu residue disrupts the normal G-protein interaction mediated through the IL4 region.

Computational modeling studies placed H8 so that it was embedded in the phospholipid head group region of the bilayer and parallel to the membrane surface [122]. From this position, L404 could extend into the membrane bilayer and interact with TM1 and the NPXXY motif. Aromatic stacking occurs in the wild-type helix between F408 and F412, both within H8, but there is no clear aromatic stacking between H8 and the transmembrane helices. Modeling of the L404F mutant, which was predicted to participate in aromatic stacking with Y397 in TM7 as occurred in rhodopsin, differed from the CB1 receptor in that the helix was rotated about 25° around the helical axis from the orientation of the CB1 receptor. In the L404I mutant, there was no aromatic stacking interaction predicted between Y397 and any residue from H8. The H8 has also undergone the rotation about its axis in this mutant. The conformational changes in both mutants results in the exposure of previously buried residues. These changes may interfere with the recognition of the receptor states by Gi3 or Go and explain the changes in biological activity.

5 G-Protein Activation Mechanisms

5.1 Conformational Changes in GPCRs

One possible mechanism for activating the coupled G protein would be the receptor conformational change leading to the destabilization of IL3 such that several key residues within the loop become actively involved in interaction with

G protein. Activation of rhodopsin was characterized by an increased mobility of IL3 due to the rearrangement of TMs 6 and 7, as indicated by the dramatic change in the distance between IL2 and IL3 [123] and by the highest B-factors of the IL3 region of rhodopsin as determined from the X-ray experiment [70, 73]. This change is believed to be from the disruption of the interhelical interaction in the TM core region, in particular between TM3 and TM6. In the inactive state, these TM helices are stabilized by H-bonding and van der Waals interactions. For examples, the X-ray structure of rhodopsin revealed that R135 in TM3 forms two H-bonds, one with E247 and the other with T251 in TM6 [73]. It appears that the flexibility of IL3 is affected greatly by any movement in the TM core. Because it is directly connected to TM5 and TM6, any change in these helices, including the highly conserved Pro residue in TM6, will influence the conformation of IL3. In fact, the impact of any conformational change in a ligand-binding site located in the extracellular side of the core will be significant upon IL3 located in the intracellular side. Thus, the resulting conformational modification in the IL regions will be important for G-protein activation.

Photoactivation of rhodopsin was shown by electron paramagnetic resonance spectroscopy to result in conformational changes throughout the receptor, including tilting of TM6 away from the core bundle relative to TM3 [123] and changes in the cytoplasmic end of TM7 and the C-terminus [59]. Fluorescence spectroscopy performed on the β_2-adrenergic receptor suggested a similar rigid-body movement between TM3 and TM6 upon agonist binding [124]. Movement of the TM6 of rhodopsin is predicted to promote a conformational change in the C-terminal of IL3, which would disrupt the stability of the receptor–G protein complex in the inactive state, leading to G-protein activation [123, 125].

It has been proposed that GPCRs contain amphipathic α-helices on the G-protein interaction surface for coupling and activation [111, 116, 126, 127]. When activated, rhodopsin appears to change its conformation such that the α-helical structure of TM5 extends to the N-terminal portion of IL3, and the α-helical structure of TM6 extends to the C-terminal end of IL3. An extension from TM6 to the distal region of IL3 [127, 128] has been reported for the α_2-adrenergic or M_4-muscarinic receptors [116]. Based upon the NMR analyses of the IL3 peptide of the rat AT_{1A} receptor in 30% trifluoroethanol, Franzoni and co-workers [71] determined a secondary structure comprising the proximal N-terminal residues 213–231 as a stable amphipathic α-helix and the distal C-terminal residues 236–241 as a more flexible conformation. These researchers suggested that the distal C-terminal part of IL3 would regulate the conformation on the proximal part of IL3 to expose the required region of the loop for G-protein coupling and activation.

Gudermann and colleagues [129, 130] reported that deletion of some residues within IL3 of thyrotropin and lutropin receptors caused these receptors to become constitutively active. They identified a distal residue D619 of thyrotropin receptor (and the equivalent D564 of lutropin receptor) as a critical residue

for maintaining the receptor in its inactive conformation through the formation of a salt bridge to another region of the receptor or to Gs. According to their proposed mechanism, the shortening of IL3 after deletion would facilitate exposing TM6 residues to the G protein for coupling [130]. Interestingly, the equivalent D567 of the follitropin receptor failed to cause constitutive activity, even though the BXXBB motif common to other glycoprotein hormone receptors was present, suggesting a functional difference within the glycoprotein hormone receptors. It was also reported that mutation of A293 at the N-terminal IL3 of the α_{1B}-adrenergic receptor by any other amino acid residue caused the receptor to be constitutively active. The authors speculated that this region would be important to constrain the G-protein coupling of the receptor [131]. As indicated by the above examples, it is likely that G-protein activation is accomplished by exposing some residues of the receptor as it changes its conformation to disrupt the receptor–G-protein (inactive) complex such that the inactive G protein is activated by the receptor to release bound GDP.

In the active state of rhodopsin, the crevice between TMs 2, 3, and 6 on the intracellular surface of the receptor is widened, due to the disruption of such interhelical interactions. With the formation of the cavity, the salt bridge between E134 and R135 in TM3 is disrupted as E134 is protonated [132] and some key residues become exposed to Gα, promoting the binding of the C-terminal residues of Gα [133]. Thus, the initial interaction between the receptor and Gα by the insertion of the C-terminal of Gα to the cavity could trigger G-protein activation by inducing a structural change in the neighboring a5/b6 domain, which would accelerate the GDP release from Gα_{GDP} [134].

5.2 *Sequential Nature of the Activation or Inverse Activation Process*

Gether and Kobilka [135] proposed a pharmacological induction model based upon progression from an initial interaction that occurs upon binding of one structural moiety of the agonist to the receptor in a ground state (R), followed sequentially by a series of further interactions via other pharmacophoric elements of the ligand. Each binding interaction would modify one or more transmembrane domains, thereby allowing a progression of conformational states of the receptor (R′ leading to R″), ultimately leading to the optimal state that maximally activates the G protein (R*). Using fluorescence spectroscopy of fluorescein-labeled proteins, Kobilka and colleagues [136, 137] determined that agonist binding induced physically distinct conformational changes of the G-protein-coupling domain of the β2-adrenergic receptor apart from the conformation found in the absence of a ligand (R) or that exhibited upon binding of an antagonist (R^o). This method was able to distinguish an intermediate conformation induced by partial (R′) and full agonists versus a relatively high-energy state that could be achieved by the full agonist (R*) [136, 137].

Fluorescence lifetime spectroscopy on the β_2-adrenergic receptor showed measurable differences in the conformations induced by binding of full versus partial agonists [136, 137]. Binding of an agonist resulted in fluorescence data that was best fit by a two-site exponential function, representing a fast and a slow phase of conformational change [138]. A series of closely related catechol compounds suggested a correlation between the conformational changes in the β_2-adrenergic receptor, structural features of the ligands, and biological activities. The data support a sequential binding model, wherein binding of a ligand proceeds through several kinetically distinct conformational states. The differences in response rates observed by fluorescence spectroscopy suggest that the different receptor states may give rise to different functional properties, such as G-protein activation and agonist-induced internalization [138]. A partial agonist, such as salbutamol, failed to interact through the same proposed rotamer toggle switch mechanism as the full agonists. The binding of antagonists did not show a conformational change between the bound and unbound states [138]. These data suggest a mechanistic model wherein several receptor conformations may be induced by the structural features of the ligands, leading to differing activity.

5.3 Steric Trigger Model

Consistent with the Kobilka model involving a series of receptor conformational changes leading to full activity [135], we propose that the agonist–CB1 receptor–G protein complex requires a sequence of transitions that must overcome a series of energy barriers in order to achieve GDP–GTP exchange and release of G proteins from the receptor. Shim and Howlett [139] described a mechanism by which the nonclassical cannabinoid CP55244 could bind to a binding pocket within the receptor in its ground state. The aminoalkylindole WIN55212-2 was able to bind in multiple loci within the binding pocket [140]. The conformation of the agonist that binds to the ground state may not be the lowest-energy conformation for that ligand. As the ligand overcomes rotational energy barriers, it achieves one or more conformations in which it can release the strain imposed by binding to the receptor's ground state [139, 140]. The ligand conversion to one or more low-energy states within the binding pocket provides a *steric trigger* to initiate a series of microconformational changes within the binding domain. Chemically distinct ligands may allow this transition to progress by multiple pathways due to their differential ability to provide the activation energy for microisomerization to unique conformations. These multiple, unique conformations can allow for the activation of selected G-protein subtypes (see [141] for discussion). Although the nonclassical cannabinoid ligand appeared to be limited in its binding locus [139], a ligand such as WIN55212-2 could bind to multiple loci and trigger more than a single conformational pathway [140]. A pathway of microconformational changes that

lead to a modification in the CB1 receptor juxtamembrane C-terminal IL4 domain would be expected to induce activation of Gαo and Gαi3 [18, 113]. In contrast, pathway of microconformational changes that lead to a modification in the CB1 receptor IL3 would be expected to be important for inducing activation of Gαi1 and Gαi2 [113]. This implies that certain agonists could induce a conformational change that is limited to the IL3, whereas others could induce alterations predominantly in the juxtamembrane C-terminal IL4. Taking advantage of this pharmacological selectivity, based upon ligand-directed responses defining selectively a specific type of G protein to be activated within cells, might be a useful strategy to propose new drug design.

Acknowledgments This work was supported by NIDA grants R01-DA03690, R01-DA06312, K05-DA00182, U24-DA12385 and K01-DA20633.

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Molecular Biology of Cannabinoid Receptors: Mutational Analyses of the CB Receptors

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Abstract Two cannabinoid receptors have been isolated by molecular cloning; there is also evidence for additional cannabinoid receptor subtypes. The CB1 and CB2 cannabinoid receptors are members of the G-protein coupled receptor (GPCR) superfamily that can be activated by plant-derived, synthetic, and endogenous cannabinoid ligands, which fall into five structurally diverse classes. Studies using mutagenesis and molecular modeling have identified key amino acid residues involved in the selective recognition, activation, desensitization, and internalization of these ligands. The CB1 and CB2 receptors genetic structures indicate polymorphisms and multiple exons that may be involved in tissue- and species-specific regulation of these genes. Polymorphisms in the CB1 and CB2 receptors are associated with drug abuse and disease. Finally, the identification of GPR55 as another GPCR that responds to a diverse set of cannabinoids may herald a new era of molecular biology of cannabinoid receptors.

Keywords Cannabinoid receptor · Mutagenesis · Polymorphism · Gene regulation · Binding

1 Introduction

The cloning and characterization of the first cannabinoid receptor in 1990 began a new era in understanding the mechanism of action of marijuana, one of the most widely used drugs throughout the world. The synthesis of novel analogs to (–)-Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary psychoactive constituent in marijuana, played a major role in the characterization and cloning of a neuronal cannabinoid receptor (CB1), a member of the G-protein

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_8,

coupled receptor family (GPCR) [1]. That the cDNA isolated was indeed a cannabinoid receptor was confirmed by transfection into CHO cells and the demonstration of cannabinoid-mediated inhibition of adenylyl cyclase [1, 2], the second messenger system that had been shown to mediate the effects of cannabinoids [3]. The CB1 receptor can also modulate G-protein coupled Ca^{2+} and K^{+} channels [4, 5] as well as extracellular signal-regulated kinases (ERK) [6].

A second type of cannabinoid receptor, the CB2 receptor, was isolated by a PCR-based strategy designed to isolate G-protein coupled receptors in differentiated myeloid cells [7]. The CB2 receptor, which until recently had only been found in the spleen and cells of immune origin (including microglia), has 44% amino acid identity with CB1, and a distinct, yet similar, binding profile, and thus represents a receptor subtype. Intriguingly, recent studies have shown CB2 localization in neurons in the brain [8, 9].

Knockout mice for each cannabinoid receptor are providing valuable information on the roles of the CB1 and CB2 receptors in vivo. The CB1 receptor gene has been inactivated in mice (by in-frame deletion of most of the coding region) using homologous recombination [10, 11]. Significantly, not only did the CB1 receptor knockout mice lose responsiveness to most cannabinoids, the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced [11]. The CB1 receptor appears to play a central role in drug addiction. The CB2 receptor gene has been inactivated by homologous recombination in mice [12]; the most notable effect was impairment of immunomodulation by helper T cells.

The discovery of endogenous ligands for the cannabinoid receptors (also in the 1990s) uncovered a novel neurotransmitter/neuromodulatory system. The first ligand, arachidonic acid ethanolamide, anandamide, was isolated from porcine brain; it competed for binding to the CB1 receptor and inhibited electrically stimulated contractions of the mouse vas deferens in the same manner as Δ^9-THC [13]. The pharmacological properties of anandamide are consistent with its initial identification as an endogenous ligand for the cannabinoid receptor(s). In vivo, anandamide produces many of the same pharmacological effects as the classical cannabinoid ligands, including hypomotility, antinociception, catalepsy, and hypothermia [14]. The biosynthetic pathways of anandamide synthesis, release, and removal are under investigation by several laboratories [15–19].

Additional fatty acid ethanolamides with cannabimimetic properties have been isolated, suggesting the existence of a family of endogenous cannabinoids [20]. 2-Arachidonylglycerol (2AG) in several systems acts as a full agonist, whereas anandamide is a partial agonist, and it has been suggested that the CB1 and CB2 receptors may in fact be 2AG receptors [21, 22].

Additional endocannabinoids have been described. Virodhamine, arachidonic acid and ethanolamine joined by an ester linkage, has been isolated [23]. N-arachidonylglycine, a conjugate of arachidonic acid and glycine present in bovine and rat brain as well as other tissues, suppresses tonic inflammatory

pain [24]. N-arachidonyl-dopamine (NADA), is primarily a vanilloid receptor agonist, but has some activity at CB1 receptors as well [25]. Palmitoylethanolamide (PEA) has been suggested as a possible endogenous ligand at the CB2 receptor [26]. Subsequent studies showed no affinity for PEA at the CB2 receptor [27–29]. Recent studies suggest that another GPCR may be responsible for PEA's actions [30].

The VR1 vanilloid receptor (TRPV1), a ligand gated ion channel that is a member of the transient receptor potential ion channel family (recently reviewed by [31]) represents an additional site of action for AEA, 2-AG, virodhamine, noladin ether, and NADA. In addition, Δ^9-THC and cannabinol at high (20 μM) concentrations have recently been identified as agonists at another TRP, the ANKTM1 channel [32]. These findings raise the possibility that the TRP channels may be ionotropic cannabinoid receptors.

This chapter will focus on the molecular biology of the G-protein coupled cannabinoid receptors.

2 General Structure, Distribution, and Pharmacology

The CB1 receptor is localized predominantly in the central nervous system (CNS), whereas the CB2 receptor is located primarily cells of immune origin. Analysis of the primary amino acid sequence of the CB1 receptor predicts seven transmembrane domain regions, typical of G-protein coupled receptors. Bramblett et al. [33] have constructed a model of the cannabinoid receptor. A representation of the CB1 receptor based on their model is shown in Fig. 1.

The human CB2 receptor has 44% amino acid sequence identity overall with the human CB1 receptor and percent similarity rises to 68% in the transmembrane domains. A representation of the CB2 receptor based on the model constructed in the Reggio lab is shown in Fig. 2. The localization of the CB2 receptor appears to be mainly in the periphery: in the spleen and in low levels in adrenal, heart, lung, prostate, uterus, pancreas, and testis and in cells of immune origin, including microglia in the CNS [7, 34, 35]; however, recent studies have provided evidence for neuronal localization of CB2 [8, 9].

Five structurally distinct classes of cannabinoid compounds have now been identified: the classical cannabinoids (Δ^9-THC, Δ^8-THC-dimethylheptyl (HU210)); nonclassical cannabinoids (CP-55,940); indoles (WIN 55,212), eicosanoids (the endogenous ligands, see below) (anandamide, 2-arachidonylglycerol); and antagonist/inverse agonists (SR141716A, SR145528) [13, 36–41].

Transfected cell lines expressing the CB1 receptor show an excellent correlation with [^{3}H]CP-55,940 binding in rat brain homogenates [37, 42, 43]. Initial studies using transfected cell lines expressing the CB2 receptor showed that many

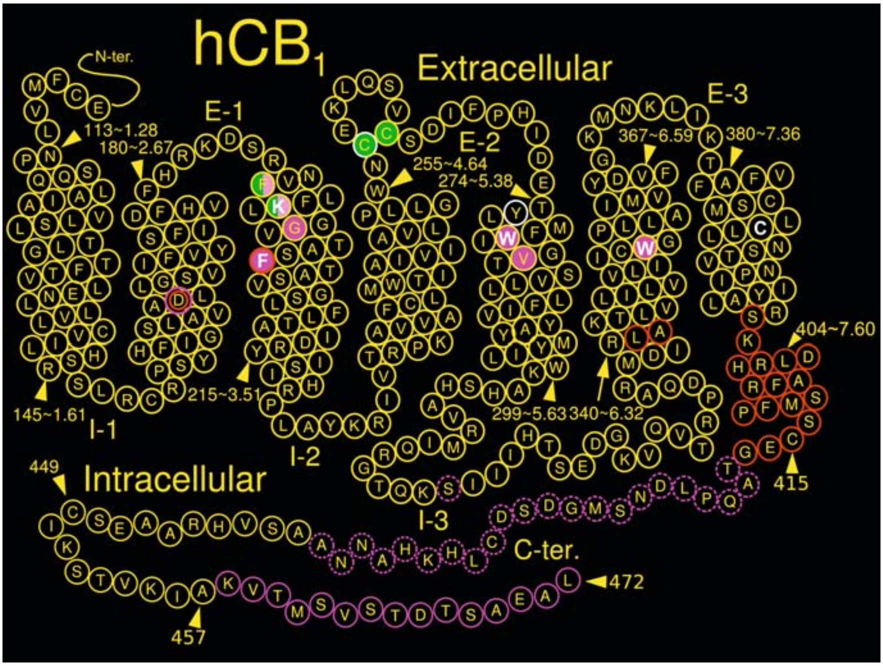

Fig. 1 A Helix Net Representation of Mutations in the CB1 Sequence. The amino acid residues important in ligand recognition for SR141716A (rimonabant) are indicated by *bold white* letters. Amino acids important for CP 55,940 binding are colored *green*. Amino acids important for WIN 55,212 binding are colored *pink*. Amino acids important for receptor activation (signal transduction) are circled in *red*. Amino acids for which all ligand binding is lost (conformational changes) are circled in *white*. Residues involved in desensitization are indicated by dotted *purple* circles. Amino acids important for internalization are circled in *purple* (*See* Color Plate 2)

cannabinoid ligands had similar affinities at both cloned receptors [7, 27, 44]. For example, the affinities for CP-55,940, Δ9-THC, 11-OH-Δ9-THC, anandamide, and cannabidiol at the CB2 receptor are comparable to the CB1 receptor. In contrast, cannabinol (which is known to be 10-times less potent than Δ9-THC at the CB1 receptor) was found to be equipotent to Δ9-THC at the CB2 receptor [7]. Additionally, WIN 55,212-2 showed a preference for the CB2 receptor [27, 44, 45]. The compounds that have been identified as CB1 and CB2 selective served as lead compounds in the design of even more selective ligands. The affinity of SR141716A (Rimonabant, Accomplia, the CB1 receptor antagonist) is at least 50-fold higher at the CB1 receptor [40] than at the CB2 receptor and provided a starting point for the design of more selective antagonists and agonists, including a CB2 receptor antagonist, SR144528 [41].

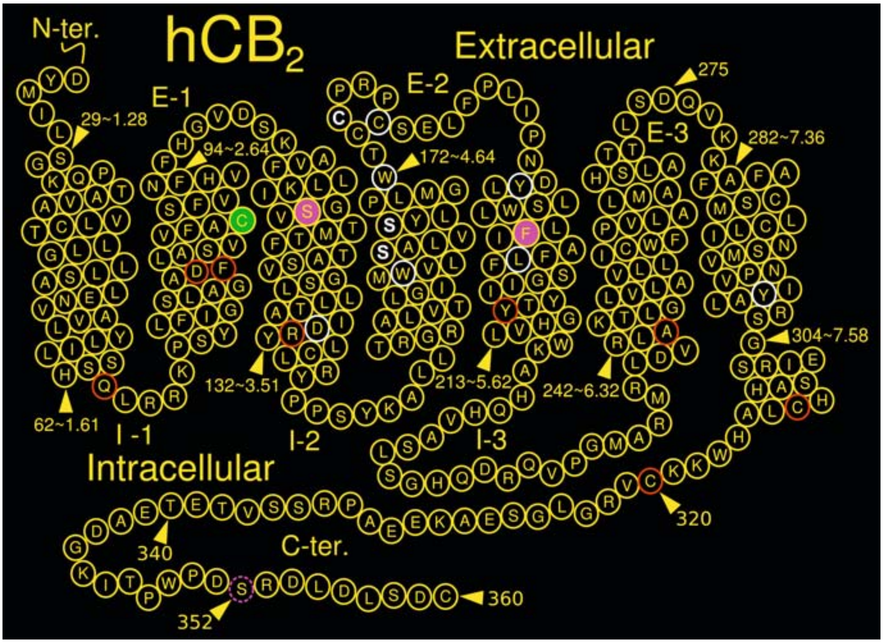

Fig. 2 Helix Net Representation of Mutations in the CB2 Sequence. The amino acid residues important in ligand recognition for SR144528 are indicated by *bold white* letters. Amino acids important for WIN 55,212 binding are colored *pink*. Amino acids important for HU 243 binding are colored *green*. Amino acids important for receptor activation (signal transduction) are circled in *red*. Amino acids for which all ligand binding is lost (conformational changes) are circled in *white*. Residues involved in desensitization are indicated by dotted *purple* circles. (*See* Color Plate 3)

3 Gene Structure and Species Diversity

The human CB1 receptor may have two distinct splice variant forms. A polymerase chain reaction amplification product was isolated that lacked 167 base pairs of the coding region of the human CB1 receptor [46]. This alternative splice form (CB1a) is unusual in that it is generated from the mRNA encoding CB1, as the coding region is contained in a single exon [47]. When expressed, the CB1a clone would translate to a receptor truncated by 61 amino acid residues with 28 amino acid residues different at the NH_2-terminal. A second splice variant of the coding region has recently been reported in which a 99 base portion of the coding exon is spliced out of the human mRNA leading to an in-frame deletion of 33 amino acids [48]. The hCB1b cDNA was isolated while cloning the previously reported splice variant. Both the CB1a and CB1b variants show altered ligand binding and GTPγS activity compared with CB1 when the cDNAs are expressed in HEK293 cells [48]. Of the six endocannabinoids

tested, only 2-AG showed significant affinity for hCB1b; furthermore, 2-AG acted as an inverse agonist at both variants. Anandamide was able to activate the variants at concentrations $>10\ \mu M$. However, THC, CP, WIN, HU210, and SR141716 exhibited good affinity and GTPγS activity with the variants. CB1a and CB1b expression has been detected in many tissues by RT-PCR [46, 48]. It will be important to confirm that the CB1a and CB1b receptor proteins expressed as splice variants often arise from incomplete splicing during library construction and RT-PCR techniques. The construction of antibodies selective to CB1 or CB1a/CB1b peptides would be useful to detect these proteins. Neither splice variant is present in rat or mouse, because the splice consensus sequence is absent in these genes [47].

Previous studies have suggested the presence of three exons upstream of the coding region of the CB1 receptor [47]. The genomic structure of the human CB1 receptor has recently been reported [49]. In this study, three exons upstream of the coding exon were identified (a total of four exons), with a variation in the first exon. Five distinct variant exonic structures were demonstrated.

The CB1 receptors are highly conserved among vertebrate species and have also been found in some invertebrates [50–52]. Shortly after the cloning of the rat cannabinoid receptor, isolation of a human CB1 receptor cDNA was reported [2]. The human CB1 receptor has one less amino acid in the N-terminus as compared to the other mammalian species (472 amino acids vs. 473 amino acids). The rat and human receptors are highly conserved, 93% identity at the nucleic acid level and 97% at the amino acid level. Similarly, the mouse and rat clones have 95% nucleic acid identity (100% amino acid identity) and the mouse and human clones have 90% nucleic acid identity (97% amino acid identity) [53–55].

A molecular phylogenetic analysis which included the CB1 receptor showed that the sequence diversity in 62 mammalian species varied from 0.41–27% [50]. In addition to mammals, the CB1 receptor has been isolated from birds [56], fish [57], amphibia [56, 58], and an invertebrate, *Ciona intestitinalis* [59]. This deuterostomian invertebrate CB receptor contains 28% amino acid identity with CB1, and 24% with CB2 [59]. Since a CB receptor ortholog has not been found in *Drosophila melanogaster* or *Caenorhabditis elegans*, it has been suggested that the ancestor of vertebrate CB1 and CB2 receptors originated in a deuterostomian invertebrate [59].

In addition to the human CB2 receptor, clones have been isolated from mouse [60, 61], rat [29, 63] and the puffer fish *Fugu rubripes* [62]. There is also information in the GenBank data on a putative zebrafish CB2 receptor. The CB2 receptor shows less homology between species than does CB1; for instance, the human and mouse CB2 receptors share 82% amino acid identity [60], and the mouse and rat 93% amino acid identity. The human, rat, and mouse sequences diverge at the C terminus; the mouse sequence is 13 amino acids shorter, whereas the rat clone is 50 amino acids longer than the human CB2 [63].

There is evidence for an intron in the C terminus of the CB2 receptor. This splice variant is also species specific; it is only present in the rat CB2 receptor [63]. This may give rise to rat-specific pharmacology of the CB2 receptor. We found differences in ligand recognition with a number of compounds at the rat CB2 receptor compared to the human CB2 receptor in transfected cells [29]. It is important to note, however, that the clone described in our studies was a genomic clone of rat CB2 and did not contain the edited C terminus described by Brown et al. [63].

In summary, from what we know so far, the diversity in the regulatory regions of the CB1 and CB2 genes may provide extensive flexibility in gene regulation of these receptors in health and disease.

4 Ligand Recognition

4.1 CB1 Receptor Binding Sites

4.1.1 The Classical/NonClassical/Endogenous CB Binding Region

Mutation studies as well as studies with novel ligands have suggested a separation of the binding site for classical (e.g., THC, HU-210), nonclassical (e.g., CP-55,940), and endogenous (e.g., anandamide) ligands from that of aminoalkylindoles (e.g., WIN 55,212) [64–66]. A K3.28(192)A mutation of CB1 resulted in greater than 1000-fold loss in affinity and efficacy for HU-210, CP 55,940, and anandamide at K3.28(192)A [65, 66]. These results indicated that K3.28(192) is a primary interaction site for the phenolic hydroxyl of HU-210 and other classical cannabinoids, as well as the nonclassical cannabinoids (e.g., CP 55,940) in the CB1 receptor [67]. Subsequent modeling studies suggested that the alkyl side chain of CP-55,940 resides in a hydrophobic pocket [64]. The primary interaction is between the phenolic hydroxyl of CP-55,940 and K3.28(192). These considerations suggest that the TMH 3-6-7 region is the binding site for classical, nonclassical cannabinoids, and presumably the endogenous cannabinoids.

However, it should be noted that the two binding regions identified (i.e., TMH 3-6-7 for other classical, nonclassical cannabinoids, and endogenous cannabinoids and TMH 3-4-5 for WIN 55,212, described below) overlap spatially such that the binding of a ligand in one region would preclude binding in the other region. Therefore, competitive inhibition by different classes of agonists would be observed in a binding assay.

Other residues in the N terminus as well as in and near extracellular loop 1 of the CB1 receptor have been found to be important for binding of CP 55,940 [68]. Dipeptide insertions were made at residues 113, 181, and 188 resulting in the loss of affinity for CP 55,940. When six substitution mutants (to alanine) were constructed around these residues, they showed weaker affinity than the WT receptor, but less of a loss than observed with the corresponding insertion

mutant. These data suggest that the loop structure itself is important for recognition of CP 55,940.

The minor sequence variation in mouse versus human CB1 can result in structural differences in ligand recognition. F189(3.25)A in human CB1 results in a dramatic reduction of CP 55,940 affinity [68], but in mouse CB1, CP 55,940 binding is not affected but instead anandamide's affinity is lowered [69].

Anandamide has also been shown to interact with F3.25 [69]. According to a molecular model of CB1, this residue is a direct interaction site for anandamide. In mutation studies, F3.25A had no effect on WIN 55,212 or SR141716A binding, but resulted in a sixfold loss in affinity for anandamide.

4.1.2 Indole and Pyrazole Antagonist Binding Region

As described above, mutation studies as well as studies with novel ligands have suggested a separation of the binding site for aminoalkylindoles (typified by WIN 55,212) from that of the other three classes of cannabinoid agonist ligands [64–66]. The K3.28(192)A mutation of CB1 results in no loss of affinity or efficacy for WIN 55,212, but greater than 1000-fold loss in affinity and efficacy for HU-210, CP 55,940, anandamide [65, 66]. In addition, a 17-fold loss of affinity is observed for SR141716A [70].

The region delimited by the fourth and fifth transmembrane domains of the CB1 receptor is crucial for the binding of the CB1 receptor antagonist SR141716A, but not CP 55,940 as shown in receptor chimera studies of the CB1 and CB2 receptors [71]. These results reinforce the hypothesis that the aminoalkylindole-binding region at the CB1 receptor is in the TMH 3-4-5 region and is not identical to that for other CB agonists. Furthermore, these results suggest that SR141716A binding shares the aminoalkylindole-binding region but also interacts with K(3.28)192.

Studies using novel ligands have shown that the carbonyl oxygen as well as the morpholino ring of the aminoalkylindoles can be replaced without affecting affinity; therefore, hydrogen bonding may not be the primary interaction of these compounds at the CB1 receptor [72–75]. Huffman et al. also reported that the replacement of the naphthyl ring of WIN 55,212 with an alkyl or alkenyl group resulted in complete loss of CB1 receptor affinity ($K_i > 10{,}000$ nM in both cases) [67]. That the carbonyl oxygen or the morpholino ring of the aminoalkylindoles can be removed without significant effect, along with evidence that the presence of the carbonyl and morpholino group (in the absence of an aryl substitutent) is insufficient to produce CB1 affinity, suggests that aromatic stacking, rather than hydrogen bonding may be the primary interaction for aminoalkylindoles at the CB1 receptor.

Modeling studies indicate that in the active state (R*) model of CB1, there is a patch of aromatic amino acids in the TMH 3-4-5 region with which WIN 55,212 can interact [69]. There is an upper (extracellular side) stack formed by F3.25 (189 in human CB1, 190 in mouse CB1), W4.64(255/256), Y5.39(275/276), and W5.43(279/280). When WIN 55,212 is computationally docked to

interact with this patch, it also can interact with a lower (toward intracellular side) aromatic residue, F3.36(200/201). In this docking position, WIN 55,212 creates a continuous aromatic stack over several turns of TMHs 3, 4, and 5 which is likely to be energetically favored. Similarly, studies in the Reggio lab suggested that in the inactive (R) state of CB1 the amide oxygen of SR141716A interacts with a salt bridge formed by K3.28 and D6.58(366), while the dichlorophenyl ring of SR141716A interacts with F3.36 and W6.48 and the monochlorophenyl ring interacts with F3.36 and W5.43 [70].

A CB1 TMH 3-4-5-6 aromatic microdomain which includes F3.25, F3.36, W4.64, Y5.39, W5.43, and W6.48 contributes to the binding domain of SR141716A and WIN 55,212 [69]. Stably transfected cell lines were created for single-point mutations of each aromatic microdomain residue to alanine. The binding of SR141716A and WIN 55,212 were found to be affected by the F3.36A, W5.43A, and W6.48A mutations, suggesting that these residues are part of the binding site for these two ligands. In particular, the W5.43A mutation resulted in profound loss of affinity for SR141716A. Mutation of W4.64 to A resulted in loss of ligand binding and signal transduction; however, this was shown to be a result of improper cellular localization; the mutant receptor was not expressed on the cell surface.

A residue in TMH7, C7.43(386) is also important for SR141716A binding [76]. Steric bulk introduced at this site, either through methanethiosulfonate labeling or by mutation (to methionine) was found to inhibit binding of SR141716A, but not CP-55940. Subsequent modeling studies suggest that this effect is caused by steric clash of the modified C386 residue with the piperidine ring of SR141716A and/or disruption of an aromatic microdomain in the binding pocket.

4.2 Ligand Recognition at the CB2 Receptor

4.2.1 SR14428 Binding Sites

A combination of site-directed mutagenesis and molecular modeling has also been used to investigate the SR144528-binding site on CB2. The first study reported that the region delimited by the fourth and fifth transmembrane domains of the CB2 receptor is crucial for the binding of WIN 55,212 and the CB2 receptor antagonist SR144528 [77]. A subsequent study showed that mutation of C175 (in the third EC loop) to serine resulted in a receptor with normal affinity for [^{3}H]CP-55,940, but loss of recognition of SR144528 [78]. Consequently, SR144528 did not act as an antagonist at this mutant. An eightfold loss of affinity for WIN 55,212 was observed with the C175S mutant. Mutation of S4.53(161) and S4.57(165) to alanines also resulted in the loss of SR144528 binding and functional activity. These serines are alanines in the CB1 receptor, which support a direct ligand-residue interaction at CB2. Several other mutations were analyzed, which did not affect SR144528 binding.

In the corresponding molecular model of CB2, SR144528 interacts with residues in TM 3,4, and 5 through a combination of hydrogen bonds and hydrophobic interactions [78]. In particular, W4.64(172) and W5.43(194) form an aromatic stack similar to that proposed for WIN 55,212 in the CB2 receptor [79] and WIN 55,212 and SR141716A in the CB1 receptor [69].

4.2.2 Amino Acids that Discriminate CB1 and CB2 Receptor Subtypes

Although the CB1 and CB2 receptors share only 44% overall amino acid identity, which rise to 68% in the transmembrane domains [7], most cannabinoid receptor agonists do not discriminate between the receptor subtypes [44, 80]. There are several ligands which are CB1- or CB2-selective (5- to 60-fold), and a few ligands with a greater separation of activity at each receptor (100- to 1000-fold) [27, 29, 64, 67, 81–84]. However, there is a need for more selective agonists to produce specific receptor-mediated effects for in vivo studies.

For example, 1-deoxy-Δ^8-THC showed no affinity for the CB1 receptor but has good affinity ($K_i = 32$ nM) for the CB2 receptor [82]. Structure–activity relationships of Δ^9-THC analogs have revealed three critical points of attachment to a receptor: (1) a free phenolic hydroxyl group; (2) an appropriate substituent at the C9 position, and (3) a lipophilic side chain [85]. However, compounds with a dimethylheptyl side chain retain affinity for both CB1 and CB2 receptors even when they lack a phenolic hydroxyl [67, 86]. Moreover, these ligands are CB2 selective [67, 82].

The ligand-binding sites of the receptors are also being mapped using in vitro mutagenesis of receptor cDNAs. The lysine residue in the third transmembrane domain of the cannabinoid receptors, which is conserved between the CB1 and CB2 receptors, appears to mediate different functional roles in the receptor subtypes. K3.28(192) in the CB1 receptor is critically important for ligand recognition for several agonists (CP-55,940, HU-210, Δ9-THC, and anandamide) but not for WIN 55,212-2 [65, 66]. Mutation of the analogous residue in the CB2 receptor (K109) to alanine or arginine resulted in fully functional CB2 receptors with all ligands tested [64]. A molecular model generated in this study suggested that an alternative binding mode could be achieved in the K109A CB2 mutant in contrast to K192A CB1. Assuming that ligand binding occurs within the pore formed by the transmembrane helix bundle, and the hydrophobic cluster of amino acids on helices 6 and 7 form the hydrophobic pocket with which the dimethylheptyl side chain of CP 55,940 interacts, receptor docking studies indicated that CP 55,940 is oriented differently in the binding pocket in CB1 versus CB2. A unique feature identified in the CP 55,940/CB2-binding site was a hydrogen-bonding cluster formed by a serine, threonine, and an asparagine. In the CP 55,940/CB1 docking studies this cluster is not present. This suggested that when CB2 K109 was mutated to A, the hydrogen-bonding cluster could compensate for receptor binding to CP 55,940, whereas when CB1 K192 was mutated to A, this compensation did not occur.

WIN 55,212 has a higher affinity for CB2 receptors, albeit only 5- to 10-fold higher than at CB1 receptors [27]. Two groups reported on critical residues in the cannabinoid receptors that may impart this agonist selectivity. Using a molecular modeling approach, which indicated that aromatic stacking interactions are important for aminoalkylindole binding, *switch* mutations were constructed in TMH5 [79]. The residue F5.46(F197) in CB2 can provide greater aromatic stacking; this residue was switched with valine (V282) as in CB1. The CB1V282F mutant showed increased affinity for WIN 55,212-2 similar to that of CB2, whereas the CB2 F197V mutant showed reduced affinity similar to that of CB1. This data strongly favored the hypothesis that F5.46 is crucial for WIN 55,212-2 selectivity. However, at the same time, the role of TM3 in WIN 55,212-2 selectivity was reported [87]. In this investigation, a CB1/CB2 chimera was constructed, CB1/2(TM3), in which the TM3 of CB1 was replaced with the corresponding region of CB2. The CB1/2(TM3) mutant bound WIN 55,212, and the other related aminoalkylindole analogs (JWH015 and JWH018), with wild-type CB2 affinities. These results suggested that the TM3 of the cannabinoid receptor imparts selectivity of aminoalkylindoles to CB2. When individual amino acid changes were evaluated, S112(3.31) in CB2 which corresponded to G195 in CB1 was the amino acid responsible for CB2 selectivity of aminoalkylindoles. Tao et al. [116] also reported that mutation of S112 in the K109AS112G mutation resulted in dramatic effects on ligand binding.

Using a combination of receptor chimeras and site-directed mutagenesis, key differences in the ligand-recognition sites of the CB1 and CB2 receptors were identified [71]. Two ligands were examined in this study, SR141716A (CB1-selective) and CP 55,940 (nonselective). Replacing the CB1 receptor with the seventh TM–C-terminal region of the CB2 receptor, including the third extracellular loop resulted in a receptor that still exhibited CB1 receptor properties. Replacing the CB1 receptor with the sixth TM–C-terminal region of the CB1-altered receptor expression; the mutant was sequestered in the intracellular compartment of the cell and could not be analyzed. Further extending the CB2 structure into the fifth and then fourth TM region of the CB1 receptor systematically resulted in a CB1/2 chimera that acted like a CB1 receptor. The fifth TM CB1/2 chimera acted as a CB1/2 hybrid and the reciprocal mutation fifth TM CB2/1 chimera had almost identical properties. The fifth TM CB1/2 chimera was similar to the wild-type CB2 receptor.

Replacing the CB1 receptor TM4-e2-TM5 region with the CB2 receptor regions resulted in a chimera that resembled the wild-type CB2 receptor [71]. These data support the hypothesis that these regions are important for CB1 receptor selectivity of SR 141716A. When just the CB1 receptor e2 domain was replaced with the CB2 receptor e2 domain, SR141716A binding was almost identical to the wild-type CB2 but in this case CP 55,940 binding was lost. A smaller sandwich chimera was also created in which just the CB1 receptor e2 domain between conserved cysteines was replaced with the corresponding CB2 receptor regions; this mutation resulted in a sequestration of the receptor.

One notable difference between cannabinoid receptors and many other GPCRs is the lack of conserved cysteines in the e2 domain; however, the e3 domain of both cannabinoid receptors does contain two or more cysteines. These cysteines are thought to form sulfhydryl bonds with cysteines in neighboring TM domains and to stabilize the receptor. When C257 and C264 in the e3 domain of CB1 receptor were replaced with serine residues the mutant receptors were sequestered [71]. These residues were then replaced with alanine. In this case the receptors were expressed normally but failed to bind CP 55,940. When cysteine residues (C174 and C179) in the e3 domain of CB2 receptor were replaced with serine residues the mutant receptor, although expressed normally on the cell surface, could not bind CP 55,940. Disruption of a disulfide bridge with the two cysteines in the amino-terminal region of the CB1 receptor was not the explanation, because the double-mutant C98,107S resulted in a receptor with wild-type properties. Overall, these results suggest that the e2 domain and corresponding cysteines are important for ligand recognition.

The role of cysteine residues in ligand recognition for the CB2 receptor was further examined in a recent cysteine-accessibility study [88]. Cysteine 2.59(89), which is located within TMH2, was found to be accessible to methanethiosulfonate ethylammonium, and furthermore, mutation of C2.59 to S reduced accessibility.

5 Receptor Activation

5.1 CB1 Receptor Constitutive Activity

Constitutive (agonist-independent) activity is observed with the overexpression of many GPCRs [89]. Experimental evidence for constitutively active CB1 receptors was first noted when SR141716A, initially described as a CB1 antagonist, was found to have inverse agonist properties [90]. Cannabinoid agonists activated MAP kinase (MAPK) activity in transfected CHO cells expressing CB1 [ibid]. However, basal MAPK activity was higher in CB1- transfected cells as compared to untransfected cells, suggesting the presence of autoactivated CB1 receptors. SR141716A not only antagonized the agonist effect on MAPK, but also reduced basal MAPK activity in CB1-transfected but not untransfected cells. Similarly, basal cAMP levels were reduced and SR141716A raised basal cAMP levels in transfected cells. The EC_{50} for SR141716A was similar to its IC_{50}, suggesting that these effects are a result of direct binding to unoccupied (precoupled) CB1 receptors and not due to the presence of endogenous ligands in the cultures. A significantly higher EC_{50} would be predicted if endogenous agonists were competing with SR141716A. Subsequent studies extended these findings to CB1 receptor-activated GTPγS binding [91] and inhibition of calcium conductance [92]. Additionally, CB1 receptors can sequester G proteins, making them unavailable to couple to other receptors [93]. SR141716A is also

an inverse agonist when CB1 receptors are coexpressed with G-protein-coupled potassium channels in *Xenopus* oocytes [5].

A study in primary cultures of rat cerebellar granule neurons presented evidence for inverse agonism by SR141716A on nitric oxide synthase activity [94]. Evidence for inverse agonism was also reported in the guinea pig small intestine [95].

Mutations (either naturally occurring or engineered) can also give rise to constitutively active GPCRs, presumably as a result of transforming the receptor to a constitutively active state. Mutations that result in constitutive activity may provide clues to the key amino acids involved in receptor activation. Generally, constitutively active receptors are also constitutively phosphorylated and desensitized, providing support for a model where a single active state conformation is the target for phosphorylation, internalization, and desensitization [96]. However, a recent study on the angiotensin II receptor and a series of studies on the CB1 receptor suggest that GPCRs may possess several transition states, each associated with conformationally distinguishable states of receptor activation and regulation [97–101].

Nie and Lewis found that the C-terminal domain contributes to constitutive activity of CB1 [102]. Truncation of the distal C-terminal tail of the CB1 receptor (at residue 417 in rat CB1) enhanced both the constitutive activity and the ability of the receptor to sequester G proteins. Conversely, mutation of a highly conserved aspartate residue in TMH2, D2.50 (164 in rat CB1) abolished G-protein sequestration and constitutive receptor activity without disrupting agonist-stimulated activity at Ca^{2+} channels. They concluded that the distal C-terminal tail acts to constrain the receptor from activating G proteins, whereas the aspartate (D2.50) in the second transmembrane domain stabilizes the receptor in both the inactive RG(GDP) state and the active R*G(GTP) state.

An interaction between F3.36/W6.48 has also been proposed to be key to the maintenance of the CB1 inactive state [103]. Previous modeling studies had suggested that an F3.36/W6.48 interaction requires a F3.36 trans $\chi1$/W6.48 g+ $\chi1$ rotameric state. SR141716A stabilizes this F3.36/W6.48 aromatic-stacking interaction, while WIN55,212-2 favors a F3.36 g+ $\chi1$/W6.48 trans $\chi1$ state [103]. McAllister et al. explored this hypothesis in a mutation study of mouse CB1 [104]. An F3.36(201)A mutation showed statistically significant increases in ligand-independent stimulation of GTPγS binding versus wild-type CB1. Basal levels for the W6.48(357)A mutant were not statistically different from wild-type CB1. F3.36(201)A demonstrated a limited activation profile in the presence of multiple agonists. In contrast, enhanced agonist activation was produced by W6.48(357)A. These results suggest that a F3.36(201)/W6.48(357)-specific contact is an important constraint for the CB1-inactive state that may need to break during activation. Modeling studies suggested that the F3.36(201)/W6.48(357) contact can exist in the inactive state of CB1 and be broken in the activated state via a χ_1 rotamer switch (F3.36(201) trans, W6.48(357) g+) $\rightarrow$ (F3.36(201) g+, W6.48(357) trans) as previously proposed.

The F3.36(201)/W6.48(357) interaction therefore may represent a *toggle switch* for activation of CB1.

5.2 CB1 Receptor Activation

Cannabinoid ligands, in addition to interacting with different amino acids in the CB1 receptor, appear to promote distinct interactions with G proteins [64, 105–110]. These different sites of ligand–receptor interaction may promote different receptor conformations, which in turn result in selective interaction with different G proteins. Evidence that different receptor conformations can promote distinct G-protein interactions was provided by a study in which a mutation produced a constitutively active CB1 receptor that coupled to G_s in preference to G_i [111]. The predominant coupling of the WT CB1 receptor is to G_i; coupling to G_s can usually only be demonstrated in the presence of pertussis toxin, which uncouples receptors from $G_{i/o}$ proteins [112]. A swap of two adjacent residues in the carboxyl terminus of the third intracellular loop/bottom of helix 6, L6.34(341)A/A6.35(342)L, resulted in a receptor that produced minimal inhibition of adenylyl cyclase in the presence of agonist, but instead showed increased basal levels of cAMP in the absence of agonist [111].

Howlett's laboratory has demonstrated using synthetic peptides derived from the CB1 receptor that the amino terminal side of the i3 loop can interact with G_i leading to the inhibition of adenylyl cyclase and that the juxtamembrane portion of the C-terminus is critical for G protein activation [113]. Synthetic peptides derived from this region can autonomously inhibit adenylyl cyclase by regulation of G_i and G_o proteins [106, 114]. C-terminal residues R400, K402, and C415 have been implicated as potential sites for G-protein activation [114]. Interestingly, the analogous region of CB2 does not activate G_i [106, 114].

As described above, the juxtamembrane carboxyl terminus region of the CB1 receptor has been shown to directly activate $G\alpha_o$ and $G\alpha_{i3}$, but not $G\alpha_{i1/2}$ proteins. We tested the hypothesis that the Leu in H8 is critically important for interaction with G proteins (unpublished observations). L7.60(404) was altered to F (as present in rhodopsin and most GPCRs) or I to add bulk . Immunoprecipitation studies revealed that both mutants couple to $G\alpha_{i1}$ and $G\alpha_{i2}$, but not to $G\alpha_{i3}$. Both mutations significantly reduced the maximal stimulation levels (Emax) of [^{35}S]GTPγS binding. The L7.60(404)I mutation significantly reduced the Emax for CP-55,940>HU-210>(+)WIN-55,212-2, while rimonabant failed to inhibit [^{35}S]GTPγS binding, consistent with loss of coupling to $G\alpha_{i3}$. The L7.60(404)F and L7.60(404)I mutant receptors internalized more rapidly than WT CB1. Electrophysiological recordings showed alterations in the ability of rimonabant to activate Ca^{2+} current. Molecular modeling suggested that in both the L7.60(404)F and L7.60(404)I mutants, the conformation of the elbow region of helix 8 (H8) (R7.56(400)-K7.58(402)) is changed and H8 (D7.59(403)-

P7.69(413)) is rotated such that the intracellular face of the TMH bundle is clearly altered from WT CB1 in the region of H8. These alterations to H8 structure may be important for differential coupling to G proteins and receptor internalization.

C-terminal residues have also been shown to be important in G-protein coupling and sequestration [99, 102, 115]. Truncation of the CB1 receptor at residue 417 attenuates G-protein coupling and truncation at residue 400 abolishes the inhibition of calcium channels produced by CB1 receptors expressed in superior cervical ganglia neurons [115]. Truncation at residue 417 also enhances constitutive activity and G-protein sequestration of receptors [102].

Mutation of D2.50(164) to N abolished G-protein sequestration and constitutive activity without disrupting agonist activity of CB1 receptors expressed in neurons [102]. The consequences of mutation of D2.50, a highly conserved residue present in most GPCRs, appear to depend on the system in which the mutant receptor is expressed. Mutation of human CB1 D2.50(163) to glutamine or glutamate-disrupted G-protein coupling but allowed the receptors to retain high affinity for cannabinoid compounds when the mutant receptors were expressed in HEK 293 cells [116]. A subsequent study by Roche et al. [100] found that rat CB1 D164N expressed in AtT20 cells retained coupling to adenylyl cyclase and inhibition of calcium currents, but did not couple to GIRK channels or internalize following cannabinoid exposure [100]. Interestingly, this same disparity had previously been observed with the α-adrenergic receptor, that transfection of D2.50 N mutant receptors into fibroblasts lacked adenylyl cyclase coupling, but those expressed in AtT20 pituitary cells coupled to adenylyl cyclase [117]. Thus, the cellular background into which the mutant receptors are introduced is also an important determinant of functional coupling. It is possible that this is due to differential localization of the transfected receptors or differential G-protein expression.

5.3 CB1 Receptor Desensitization and Internalization

Regions of the CB1 receptor involved in receptor regulation following chronic agonist exposure have been identified. Rapid internalization of CB1 receptors was observed after agonist exposure [101, 118]. In contrast, chronic treatment of cells with the inverse agonist SR141716A caused upregulation of cell surface receptors [118]. As in other GPCRs, the C-terminal domain is critical for receptor internalization; truncation of the terminal 14 amino acids eliminates receptor internalization [101]. Truncation of the C terminus at residue 418 abolished desensitization, as did deletion of residues 418–439 [99].

On the other hand, phosphorylation of S426 and S430 (tail region) or S317 (third intracellular loop) resulted in CB1 receptor desensitization, however, these sites had no influence on internalization [99, 119]. While receptor

internalization was not affected when G-protein signaling was disrupted by treatment with pertussis toxin, a mutation of the highly conserved aspartate residue in the second transmembrane domain in which G-protein coupling is altered did block CB1 receptor internalization [100].

We recently discovered that L7.60(404) also plays a role in receptor internalization [120]. The L7.60(404)F and L7.60(404)I mutant receptors internalized more rapidly than WT CB1. Because the intracellular face of the mutant receptors was predicted to be altered, this topographical change could also influence the interactions between the mutant receptors and the GRKs and/or β-arrestins. In summary, the mutations which alter the rate of CB1 receptor internalization may disclose mechanisms involved in tolerance toward cannabinoids.

5.4 CB2 Constitutive Activity

Constitutive activity has also been shown with the CB2 receptor [121]. CB2 receptors expressed in CHO cells also sequester G_i proteins; the CB2 inverse agonist SR144528 inhibits basal G-protein activity as well as switching off MAPK activation from receptor tyrosine kinases and the GPCR LPA receptor [121]. CB2 receptors are constitutively phosphorylated and internalized [122]. Autophosphorylation as well as agonist-induced phosphorylation occurs on S352 and involves a G-protein-coupled receptor kinase (GRK) [122].

5.5 CB2 Receptor Activation

One of the first mutation studies with the CB2 receptor examined mutation of the highly conserved aspartate residue in the second transmembrane domain of the CB2 receptor, D2.50(80) [116]. As with the CB1 receptor, mutation of aspartate to glutamine or glutamate disrupted G-protein coupling without affecting high-affinity agonist binding [116].

A *DRY* motif is important for activation of a number of GPCRs. This motif has been examined in two separate studies of the CB2 receptor, with different results [123, 124]. Both investigations found that mutation of D3.49(130) to A resulted in loss of ligand binding and subsequent signal transduction [123, 124]. This was proposed to be due to a conformational change in the CB2 receptor, rather than a direct effect on ligand binding, since this residue is at the cytoplasmic end of TM3. Mutation of Y2.51(132) to A resulted in a loss of signal transduction without affecting ligand recognition [124]. However, Rhee et al. [124] demonstrated that mutation of R3.50(131) to A resulted in a slight reduction of signal transduction, whereas Feng and Song [123] found no evidence for G-protein coupling in the mutant receptor, including an abolition

of constitutive activity in the mutant cell line. In one case, transient transfection into COS cells was employed [124], in the other case, stable transfection into HEK 293 was used [123], again suggesting that the cellular background plays an important role in the function of these GPCRs. Coupling to different G proteins is one explanation for the disparate results. In fact, a recent study found that 2-AG induced a pertussis toxin-sensitive response, whereas CP 55,940 functional responses were unaffected by treatment with pertussis toxin; mutation of R3.50(131) to A resulted in reduction of the 2-AG but not the CP55,940-mediated responses [125].

Feng et al. also examined mutation of A6.34(244) to glutamate [123]. The A6.32(244)E mutation resulted in a loss of ligand binding, signal transduction, and constitutive activity. The location of this amino acid, at the bottom of helix 6 suggests that it may be important in receptor conformation. Highlighting the differences between CB1 and CB2 receptors, this amino acid in the CB1 receptor was partly responsible for enhancing G-protein coupling to Gs [111].

A tyrosine residue conserved between CB1 and CB2, Y5.58(207), is critical for signal transduction in the CB2 receptor [126]. The Y5.58A mutant receptor retained ligand binding, albeit with an eightfold reduced affinity for [^{3}H] WIN-55,212, and fivefold reduction in HU-210 and anandamide binding. This residue resides at the cytoplasmic end of helix 5, an area which has been demonstrated to be involved in G-protein coupling; therefore, this conserved tyrosine may play a role in propagation of agonist-induced conformational changes for signal transduction [126].

Cysteine residues in the C-terminal domains have been shown to be important in functional coupling in several GPCRs. Mutation of C313 or C320 to alanine in the CB2 receptor resulted in a mutant that retained WT ligand recognition properties but loss of functional coupling to adenylyl cyclase [127]. In several other GPCRs, C-terminal cysteine mutations also lead to lack of desensitization; this was not the case with the CB2 receptor [127]. These data demonstrate the importance of residues in the C-terminal domain to functional coupling in the CB2 receptor.

5.6 CB2 Receptor Desensitization and Internalization

The CB2 receptor is desensitized and internalized following agonist treatment in vitro [122]. Studies conducted in CB2-transfected CHO cells demonstrated that phosphorylation at S352 appears to play a key role in the loss of responsiveness of the CB2 receptor. Furthermore, SR144528 could regenerate the desensitized CB2 receptors by activating a phosphatase that dephosphorylated the receptor. Hence the pharmacological properties and phosphorylation state of the CB2 receptor can be regulated by both agonists and antagonists.

5.7 Receptor Conformation

Several residues have been found to be important for maintaining proper receptor conformation for ligand recognition. For example, at the top of the TMH 3-4-5 aromatic cluster in both the CB1 (Y5.39(275)) and CB2 (Y5.39(190)) receptors is a tyrosine residue. Creating a tyrosine to phenylalanine mutation in both CB1 and CB2 resulted in subtle alterations in receptor affinity and signal transduction. In contrast, a tyrosine to isoleucine mutation in CB1 and CB2 led to receptors which lost ligand-binding capability [128]. Evaluation of receptor expression revealed no significant differences between the Y5.39I mutant and the wild-type receptor. Mutation of Y5.39(275) to A resulted in a receptor which failed to be expressed at the cell surface [77]. Monte Carlo/Stochastic Dynamics studies suggested the hypothesis that aromaticity at position 5.39(275) in CB1 and 5.39(190) in CB2 is essential to maintain cannabinoid ligand wild-type affinity; while the CB1 Y5.39(275)F mutant was very similar to WT, the Y5.39(275)I mutant showed pronounced topology changes in the TMH 3-4-5 region [128].

Rhee et al. found that two conserved tryptophan residues, W4.50(158) and W4.64(172), are required for proper ligand recognition and signal transduction [129]. W4.50 is conserved among most GPCRs, whereas W4.64 is conserved between CB1 and CB2 receptors. Substitutions to aromatic residues phenylalanine or tyrosine as well as to leucine and alanine were evaluated. For both tryptophan residues, the W to F mutant retained WT binding and signaling properties and the L and A mutations resulted in loss of ligand binding and signal transduction. In this study, expression of protein was assessed by Western analyses; however, cellular localization was not examined [129]. W4.64 has been suggested to be an interaction site for the aminoalklyindoles and pyrazole anatagonists, and in CB1, the W4.64A mutation resulted in a receptor that did not localize to the cell surface [128].

In most GPCRs, there is a proline residue in the middle of TM5, but in the cannabinoid receptors, this residue is a leucine. Substitution of L5.50(201) to proline caused a complete loss of ligand binding and function, probably due to an overall conformational change in the mutant receptor [126].

The highly conserved tyrosine in the NP$(X)_n$Y motif in TM7 also plays an important role in the CB2 receptor's proper conformation for ligand recognition and signal transduction [127]. The Y7.53(299)A mutation produced a receptor that was correctly targeted to the cell membrane, yet led to a complete loss of ligand binding and functional coupling to adenylyl cyclase. Since the location of Y7.53(299) is very close to the cytoplasmic face, it is not postulated to be directly involved in ligand binding; instead these results are probably due to conformational changes in the receptor protein [127].

6 Receptor Polymorphisms in Addiction and Disease

6.1 CB1 Receptor Polymorphisms

As the CB1 receptor has been shown to regulate cocaine and heroin reinforcement as well as opioid dependence [11, 130], polymorphisms in CB1 may play a role in drug addiction. When the CB1 receptor was knocked out by homologous recombination, not only did the mutant mice lose responsiveness to cannabinoids, the reinforcing properties of morphine and the severity of the withdrawal syndrome were strongly reduced [11]. Several laboratories have demonstrated that CB1 receptors regulate mesolimbic dopaminergic transmission in brain areas known to be involved in the reinforcing effects of morphine and it has now been shown that the CB1 receptor is critical for this μ opioid receptor effect [131–133]. In addition to increasing mesolimbic dopamine, Δ^9-THC facilitates brain-stimulation-reward, an animal model for abuse liability [134]. Moreover, genetic variations in the response have been clearly demonstrated in three strains of rats [135]. Lewis rats showed the most pronounced Δ^9-THC-induced enhancement of brain-reward functions. Sprague-Dawley rats showed an enhancement that was approximately half that seen in Lewis rats. And at the dose-tested, brain-reward functions in Fischer 344 rats were unaffected. A subsequent study also found a strain-specific facilitatory effect on dopamine efflux in nucleus accumbens [136]. These data demonstrate that genetic variations to cannabinoid effects exist and suggest that genetic variation influences drug abuse vulnerability. Indeed, differential sensitivity to Δ^9-THC in the elevated plus-maze test of anxiety was also shown in three mouse strains [137]. Two different doses of Δ^9-THC induced aversion to the open arms of the maze in ICR mice but not in DBA/2 and C57BL/6 mice. Basal locomotor activity was significantly different in the three strains of mice, and may be related to differences in CB1 receptor function [138].

CB1 receptor polymorphisms may underlie differential sensitivity to Δ^9-THC. The CB1 receptor has been cloned and sequenced from two strains of mice, C57BL/6 [54] and 129SJ [53], as well as from NG108-15 cells [55]. Additional mouse genomic sequence information has been deposited at NCBI. However, the additional full-length sequences are also from the 129SJ strain. Sequence analysis of the C57BL/6 CB1 receptor cDNA (accession #U17985), indicates three amino acid differences compared to that obtained from the 129SJ strain (genomic clones, accession # U22948) and NG108-15 (cDNA clone, accession #U40709) [53]. One of them, T210R, is in the third transmembrane domain, an area found to be critical for ligand recognition in the CB1 receptor [64–66, 87]. In addition, a recent report showed distinct differences in CB1 receptor binding properties in the brains of C57Bl/6 and DBA/2 mice [139]. It is possible that naturally occurring mutations confer functional differences in CB1 responses.

Several human CB1 receptor polymorphisms have been identified. The initial polymorphism found was a restriction fragment length polymorphism (RFLP) in the intron preceding the coding exon of the receptor [140]. The CB1 receptor gene is intronless in its coding region, but possesses an intron 5′ to the coding exon with three putative upstream exons [47, 49].

A positive association between a microsatellite polymorphism ($(AAT)_n$) in the CB1 gene and IV drug abuse has been described [141]. This polymorphism has subsequently been localized 3′ to the coding exon of the CB1 receptor [49]. Although there are differences between populations, the CB1 $(AAT)_n$ polymorphism has also been associated with schizophrenia [142], as well as with depression in Parkinson's disease [143], providing genetic evidence for a role of the cannabinoid system in these disorders.

The first polymorphism in the coding exon described was a silent mutation in T453 (G to A), a conserved amino acid present in the C-terminal region of the CB1 and CB2 receptors, that was a common polymorphism in the German population [144]. While this mutation is silent, analysis of several human sequences present in the database reveals that CB1K5 (accession #AF107262), a full length sequence, contains five nucleotide changes, three of which result in amino acid differences. Coincidentally, two amino acid differences are in the third transmembrane domain, F200L and I216V. The third variant is in the fourth transmembrane domain, V246A. A recent report by the group that submitted the sequence to the database revealed that this was a somatic mutation in an epilepsy patient; that is, DNA obtained from their blood was unaltered, but DNA from the hippocampus showed the mutation [145]. The presence of a somatic mutation rather than a polymorphism is generally indicative of the disease process in cancers (e.g., mutant p53 or APC expression in tumors but not normal tissues [146, 147]). CB1 receptor polymorphisms may affect responsiveness to cannabinoids.

Zhang et al. studied several polymorphisms in control and drug-abusing individuals from European, African, and Japanese ethnicities and found association with a 5′ *TAG* haplotype that was highly associated with substance abuse in all three populations [49]. Analysis of mRNA levels from postmortem brain samples of individuals with the TAG haplotype showed reduced expression for individuals expressing this allele. In sum, the genomic studies implicate the CB1 receptor in drug addiction and disease.

6.2 CB2 Receptor Polymorphisms

Polymorphisms in the CB2 receptor have recently been identified [148, 149]. As both the human and mouse CB2 genes map to areas associated with osteoporosis and bone density, Karsak et al. compared 220 osteoporetic postmenopausal women compared to 160 controls, and found a significant association with several haplotypes within the CB2 gene [148]. Among the large number of

associated polymorphisms, two were missense variants; Q63(1.62)R and H316(7.70)Y. These alterations, alone or in combination with other linked polymorphisms, may alter protein function and/or gene expression. Indeed, CB2 knockout mice show enhanced bone loss and a CB2 agonist slows ovariectomy-induced bone loss [150]. Furthermore, the Q63R polymorphism has been shown to affect endocannabinoid action in lymphocytes (see paragraph below). These data suggest that the CB2 receptor may play an important role in osteoporosis that may be amenable to pharmacologic treatment.

As the CB2 receptor may play a role in immunomodulation, Sipe et al. sought to identify polymorphisms in the CB2 gene [149]. They identified a common polymorphism, 188–189 GG/GG which predicts the substitution of Q by R at amino acid 63. This is one of the polymorphisms identified in the osteoporosis study described above. A higher incidence of the GG/GG phenotype was found in 102 Caucausian autommine patients (~40%) compared with 519 Caucasian controls (~30%). T lymphocytes from GG/GG homozygote patients showed reduction in 2-AG inhibition of CD3-stimulated T-cell proliferation, and SR144528, the CB2 receptor antagonist, provided partial reversal of this reduced response. These data suggest that the CB2 receptor may play an important role in immune dysfunction that may be amenable to pharmacologic treatment.

7 Evidence for Additional Cannabinoid Receptor Subtypes

There is compelling evidence for the existence of additional cannabinoid receptor subtypes. Early studies performed after the identification of CB1 and CB2 receptors suggested that anandamide inhibits gap-junction conductance and intercellular signaling in striatal astrocytes via a CB1 receptor-independent mechanism; since the cannabimimetic agents CP-55,940 and WIN-55,212 did not mimic the effect of anandamide, nor did the CB1 receptor antagonist SR141716A reverse anandamide’s actions [151].

Studies using CB1 receptor knockout mice, which have now been constructed in four laboratories [10, 11, 83, 152], also point to the existence of receptor subtypes. In one strain, although CB1 receptor knockout mice lost responsiveness to most cannabinoids, Δ^9-THC still produced antinociception in the tail-flick test of analgesia [10]. Further characterization of this non-CB1 Δ^9-THC response suggests the presence of a novel cannabinoid receptor/ion channel in the pain pathway [153].

Evidence for an anandamide- and WIN 55,212-sensitive cannabinoid receptor subtype has been provided by studies using two different strains of knockout mice. Anandamide produces the full range of behavioral effects (antinociception, catalepsy, and impaired locomotor activity) in CB1 receptor knockout mice [154]. Futhermore, anandamide-stimulated GTPγS activity can be elicited in brain membranes from these mice [155]. These effects were not sensitive to

inhibition by SR141716A. Interestingly, of all cannabinoid ligands tested, only WIN 55,212 elicited GTPγS activity in CB1 knockout mice. This same phenomenon has also been demonstrated in a second strain of CB1 receptor knockout mice [156].

A different cannabinoid receptor subtype has been pharmacologically identified in the hippocampus that is responsive to WIN 55,212 and CP 55,940 and blocked by capsazepine [157]. These receptors are found on excitatory (pyramidal) axon terminals and have been shown to suppress glutamate release in CB1 receptor knockout animals. Further characterization of this response led Hoffman et al. to conclude that this receptor may be strain specific [158].

In addition, an *abnormal cannabidiol receptor* has also been characterized. Cannabinoids including anandamide elicit cardiovascular effects via peripherally located CB1 receptors [159–161]. Abnormal cannabidiol (abn-cbd), a neurobehaviorally inactive cannabinoid that does not bind to CB1 receptors, caused hypotension and mesenteric vasodilation in wild-type mice as well as in mice lacking CB1 receptors or both CB1 and CB2 receptors [160]. In contrast to the studies described above, these cardiovascular and endothelial effects were SR141716A sensitive. A stable analog of AEA (methanandamide) also produced SR141716A-sensitive hypotension in CB1/CB2 knockout mice. These effects were not due to activation of vanilloid receptors, which also interact with AEA [162]. A selective antagonist, O-1918, has recently been developed; it inhibits the vasorelaxant effects of abn-cbd and anandamide [163].

Signal transduction pathways for the abn-cbd receptor have been studied in human umbilical endothelial cells (HUVEC) [163]. Abn-cbd induces phosphorylation of ERK and protein kinase B/Akt via a PI3 kinase-dependent, pertussis toxin-sensitive pathway; these effects were blocked by O-1918 [163]. The abn-cbd receptor subtype also appears to be present in microglia [35]. Anandamide and 2-AG triggered migration in BV-2 cells, a microglial cell line; their effects were blocked with O-1918. 2-AG also induced phosphorylation of ERK1/2 in BV-2 cells [35]. These data suggest a common signaling pathway for the abn-cbd receptor in endothelial cells and microglia.

Palmitoylethanolamide has been suggested as a possible endogenous ligand at the CB2 receptor [26]. However, it has a low affinity for the cloned human CB2 receptor [27]. This difference suggested that there may be species differences with the CB2 receptor, as have been found with other G-protein-coupled receptors, but the cloned rat and mouse CB2 receptors also showed low affinity for palmitoylethanolamide [29]. Palmitoylethanolamide has recently been shown produce to a G-protein-mediated response in microglial cells that was not affected by CB1, CB2, or abn-cbd antagonists, suggesting it acts via its own GPCR [30].

Finally, recent studies indicate that a previously identified *orphan* GPCR, GPR55, is a cannabinoid receptor [164–166]. GPR55 was isolated in 1999 as an orphan GPCR with high levels of expression in human striatum [167]. Its ability to recognize cannabinoids was first described in a yeast expression system, where the CB1 antagonists AM251 and SR141716A (rimonabant) acted as

agonists at micromolar concentrations [165]. However, Sjogren et al. have expressed GPR55 with Gα13 in HEK293 cells; there nanomolar concentrations of many cannabinoid agonists stimulated GTPγS binding [166]. Most of the endocannabinoids, including anandamide, 2-AG, virodhamine, noladin ether, and palmitoylethanolamine, as well as the agonists CP55,950 and Δ^9-THC, stimulated GTPγS binding, which was not antagonized by AM281, but was blocked with 450 nM abn-CBD [166]. AM251 produced an agonist response in HEK293 cells, similar to that found in the yeast expression system. However WIN55,212 did not produce an agonist response at GPR55, indicating that this is a distinct receptor from the anandamide- and WIN-sensitive receptor described by Di Marzo et al. [154].

In summary, there is compelling evidence for the existence of additional cannabinoid receptor subtypes. Most convincingly, GPR55 appears to be a novel cannabinoid receptor, although several other putative subtypes have been characterized pharmacologically.

8 Conclusion

That there is a complex molecular architecture of the cannabinoid receptors has become apparent from the growing number of mutagenesis investigations, synthesis of CB1- and CB2-selective compounds, and discovery of multiple endogenous agonists, as well as identification of new receptor subtypes. A single receptor can recognize multiple classes of compounds and produce an array of distinct downstream effects. Natural polymorphisms and alternative splice variants may also contribute to the pharmacological diversity of the cannabinoid receptors. As our knowledge of the distinct differences grows, we may be able to target select receptor conformations and their corresponding pharmacological responses. Additional receptor subtypes will add to the complexity of the endocannabinoid system, the basic biology of which will continue to be revealed by ongoing investigations.

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Models of Cannabinoid Inverse Agonism, Neutral Antagonism, and Agonism: Tools for Rational Drug Design

Dow P. Hurst and Patricia H. Reggio

Abstract The cannabinoid receptors belong to Class A of the G protein-coupled receptor (GPCR) family. GPCRs are assumed to have a common topology and to share a common molecular activation mechanism involving their intracellular domains. However, each individual receptor will also have a molecular switch within the ligand-binding pocket that is a noncovalent, intramolecular interaction in the basal state of the GPCR that must be disrupted to achieve an active state. Knowledge of the molecular switch within the ligand-binding pocket can greatly facilitate the rational design of inverse agonists, neutral antagonists, and agonists.

This chapter reviews the experimental literature on GPCR structure and activation and then focuses on sequence divergences between the cannabinoid CB1/CB2 receptors and other Class A GPCRs that may cause CB1 and CB2 to diverge from the structure of the prototypical Class A GPCR, rhodospin. Results of computer modeling, mutation, and covalent labeling studies are presented that have led to our current understanding of cannabinoid CB1 and CB2 receptor structure and activation at a molecular level. An outgrowth of these studies has been the identification of the CB1 receptor F3.36/W6.48 molecular toggle switch. The design of compounds that stabilizes this toggle switch (inverse agonists) versus those that disrupt the switch (agonists) are presented by examining the interaction of each cannabinoid ligand structural class at their receptors.

Keywords Inverse agonist · Neutral antagonist · Agonist · Allosteric modulator · G protein-coupled receptor

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_9,

1 Introduction

G protein-coupled receptors (GPCRs) are membrane proteins that serve as very important links through which cellular signal transduction mechanisms are activated. Many vital physiological events such as sensory perception, immune defense, cell communication, chemotaxis, and neurotransmission are mediated by GPCRs. Not surprisingly, GPCRs are major targets for drug development today. Both the cannabinoid CB1 and the CB2 receptors belong to the Class A, rhodopsin-like family of GPCRs.

1.1 The CB1 Receptor

The cloning and expression of a complementary DNA from a rat cerebral cortex cDNA library that encoded the first cannabinoid receptor subtype (CB1) was reported by L.A. Matsuda and co-workers [1]. Subsequently, the primary amino acid sequences of an amino terminus variant CB1 receptor [2], as well as the CB1 sequence in human brain and mouse were reported [3, 4]. A helix net representation of the human CB1 receptor sequence is presented in Fig. 1. In addition to being found in the central nervous system (CNS), mRNA for CB1 has also been identified in testes [3]. The CB1 receptor has been shown to have a high level of ligand-independent activation (i.e., constitutive activity) in transfected cell lines, as well in cells that naturally express the CB1 receptor

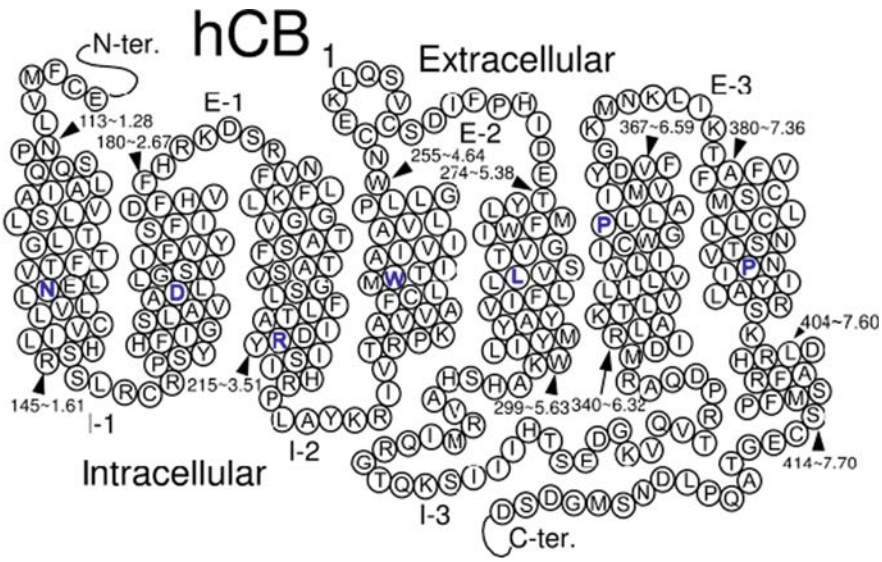


Fig. 1 A helix net representation of the human CB1 receptor is presented here. The most highly conserved residue across Class A GPCRs in each helix is highlighted in bold

[5–8]. Kearn and co-workers [9] have estimated that in a population of WT CB1 receptors, 70% exist in the inactive state (R) and 30% exist in the activated state (R*). The CB1 receptor has an exceptionally long extracellular N-terminal domain (N-tail) of 116 amino acids, but has no typical signal sequence. Andersson and co-workers [10] have reported that the long N-tail cannot be efficiently translocated across the endoplasmic reticulum (ER) membrane, causing the rapid degradation of CB1 by proteasomes and leading to a low level of expression of the receptor at the plasma membrane. These investigators have proposed that the N-tail may play an important role in regulating the stability and surface expression of CB1.

1.2 The CB2 Receptor

The second cannabinoid receptor subtype, CB2, was derived from a human promyelocytic leukemia cell HL60 cDNA library [11]. The human CB2 receptor exhibits 68% identity to the human CB1 receptor within the transmembrane regions, and 44% identity throughout the whole protein. The CB2 receptor in both rat [12] and mouse [13] has been cloned as well. A helix net representation of the human CB2 receptor sequence is presented in Fig. 2. Unlike the CB1 receptor, which is highly conserved across human, rat, and mouse, the CB2 receptor is much more divergent. Sequence analysis of the coding region of the rat CB2 genomic clone indicates 93% amino acid identity

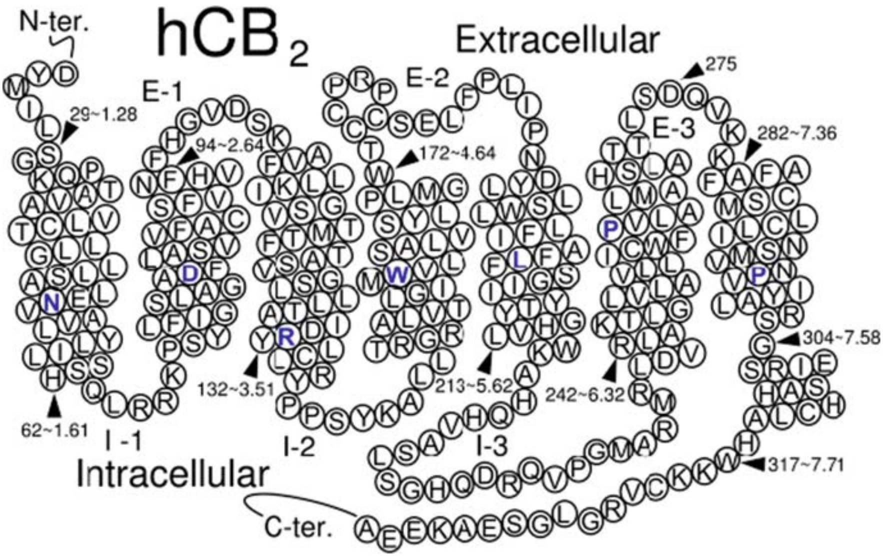


Fig. 2 A helix net representation of the human CB2 receptor is presented here. The most highly conserved residue across Class A GPCRs in each helix is highlighted in bold

between rat and mouse and 81% amino acid identity between rat and human. CB2 receptor-transfected Chinese hamster ovary cells exhibit high constitutive activity [14].

1.3 A New Putative Cannabinoid Receptor

A large number of physiological processes are controlled by the endogenous cannabinoid system [15]. Most of these effects have been attributed to action at either the CB1 or CB2 cannabinoid receptors. Yet there are effects that clearly are not CB1 or CB2 mediated [16]. Some of these cannabinoid effects may in fact not be receptor mediated at all. However, there is sufficient evidence to suggest the involvement of the TRPV1 (vanilloid) receptor in mediating cannabinoid effects and at least two other cannabinoid receptor subtypes defined only pharmacologically thus far [16]. Very recently, a Class A orphan GPCR, GPR55, has been suggested to be a cannabinoid receptor, [17] with a fairly wide range of cannabinoid ligands reported to display affinity/efficacy. GPR55 has been identified as a putative cannabinoid receptor in three recent abstracts [18–20] as well as in prior patent applications [21, 22]. These patents can be found on the internet (e.g., http://ep.espacenet.com). GPR55 was originally isolated in 1999 as an orphan GPCR with high levels of expression in human striatum [23] (Genbank accession # NM_005683). Drmota and co-workers have also isolated a variant of GPR55, GPR55a, which contains three amino acid substitutions (F3.33(102)L, G5.52(195)S, C7.47(281)R) [22]. Lysophosphatidyl inositol has been reported to activate GPR55 at μM concentrations [24].

1.4 Cannabinoid Receptor Ligands

1.4.1 Cannabinoid Agonists

The CB1 receptor transduces signals in response to CNS-active constituents of *Cannabis sativa*, such as the classical cannabinoid (–)-trans-delta-9-tetrahydrocannabinol ((–)-Δ^9-THC (**1**; Fig. 3)) and to three other structural classes of ligands, the nonclassical cannabinoids typified by (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl) cyclohexan-1-ol (CP55940 (**2**; Fig. 3)) [25, 26], the aminoalkylindoles (AAIs) typified by (R)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone (WIN55212-2 (**3**; Fig. 3)) [27–29] and the endogenous cannabinoids. The nonclassical cannabinoids (CBs) clearly share many structural features with the classical cannabinoids, for example, a phenolic hydroxyl at C-1 (C2′), and alkyl side chain at C-3 (C-4′), as well as the ability to adopt the same

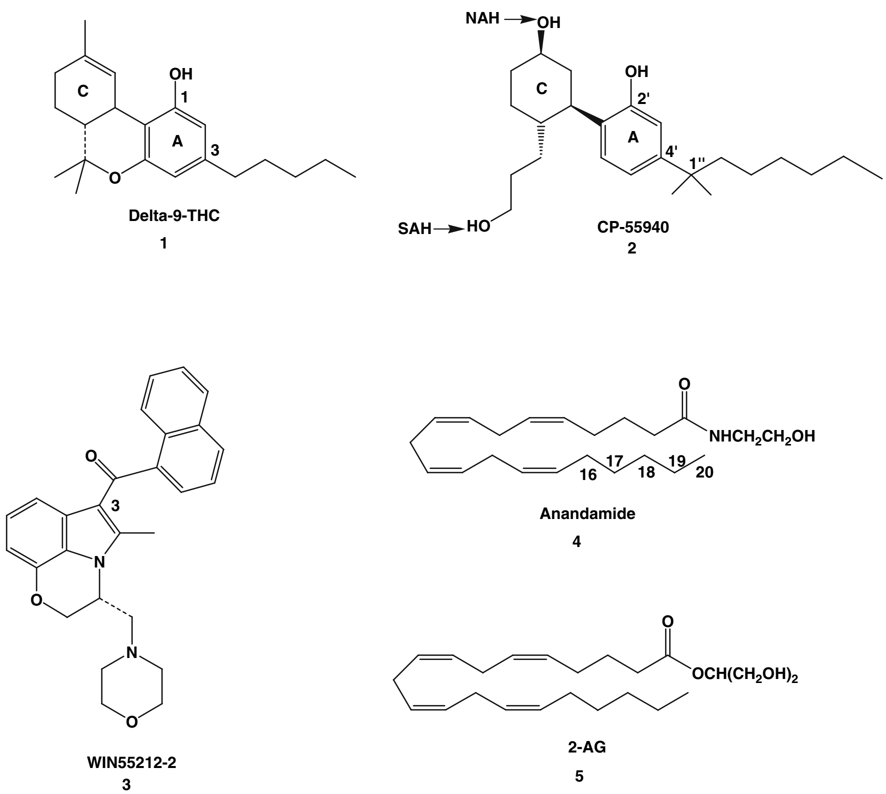


Fig. 3 Structures of cannabinoid agonists from each structural class are illustrated here

orientation of the carbocyclic ring as that in classical cannabinoids [30]. The AAIs, on the other hand, bear no obvious structural similarities with the classical/nonclassical cannabinoids.

The first endogenous cannabinoid was isolated from porcine brain by Mechoulam and co-workers [31]. The endogenous cannabinoid ligands are unsaturated fatty acid ethanolamides. The first identified ligand of this class was arachidonylethanolamide (AEA, also called anandamide, **4;** Fig. 4) [31]. AEA has been shown to be synthesized from lipid in neurons and to be degraded by fatty acid amide hydrolase, FAAH, an integral membrane protein [32]. Sn-2-arachidonylglycerol (2-AG; **5;** Fig. 4) was isolated from intestinal tissue and shown to be a second endogenous CB ligand (CB1 $K_i = 472 \pm 55$ nM; CB2 $K_i = 1400 \pm 172$ nM) [33]. 2-AG has been found present in the brain at concentrations 170 times greater than AEA [34]. In addition, fatty acid glycerol ether, 2-arachidonyl glyceryl ether, called noladin ether, has been identified as another endogenous cannabinoid ligand [35].

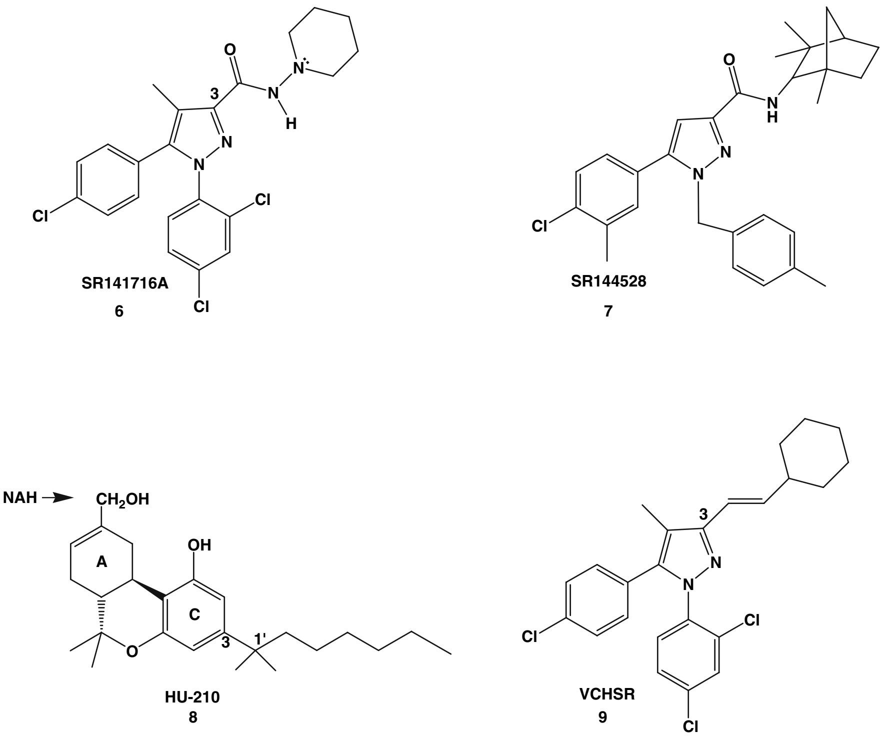


Fig. 4 Structures of a cannabinoid CB1 inverse agonist (**6**), a cannabinoid CB2 inverse agonist (**7**), a cannabinoid agonist (**8**), and a cannabinoid neutral antagonist (**9**) are illustrated here

1.4.2 Cannabinoid Receptor Antagonists/Inverse Agonists

The first CB1 antagonist, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A (**6;** Fig. 4)) was developed by M. Rinaldi-Carmona and co-workers at Sanofi Recherche [36]. SR141716A displays nanomolar CB1 affinity (Ki = 1.98 ± 0.13 nM), but very low affinity for CB2. In vitro, SR141716A antagonizes the inhibitory effects of cannabinoid agonists on both mouse vas deferens (MVD) contractions and adenylyl cyclase activity in rat brain membranes. SR141716A also antagonizes the pharmacological and behavioral effects produced by CB1 agonists after interperitoneal (IP) or oral administration [36]. Several other CB1 antagonists have been reported, LY-320135 [37], O-1184 [38], CP-272871 [7], a class of benzocycloheptapyrazoles [39], and a novel series of 3,4-diarylpyrazolines [40].

SR141716A [7] has been shown to act as a competitive antagonist and inverse agonist in host cells transfected with exogenous CB1 receptor, as well as in biological preparations endogenously expressing CB1. Bouaboula and co-workers [5] reported that CHO cells transfected with human CB1 receptor exhibit high constitutive activity at both levels of MAP kinase and adenylyl

cyclase. Guanine nucleotides enhanced the binding of SR141716A, a property of inverse agonists. Lewis and co-workers [6] demonstrated constitutive activity of CB1 receptors in inhibiting Ca^{2+} currents that was not due to endogenous agonist. These investigators reported that SR141716A antagonized the Ca^{2+} current inhibition induced by the cannabinoid agonist, WIN-55212-2, in neurons heterologously expressing either rat or human CB1 receptors. Further, when applied alone, SR141716A increased the Ca^{2+} current, with an EC_{50} of 32 nM, via a pertussis toxin-sensitive pathway, indicating that SR141716A can act as an inverse agonist by reversal of tonic CB1 receptor activity. Howlett and co-workers [7] demonstrated that constitutive activity is demonstrable in neuronal cells that endogenously express CB1 (N18TG2 cells) and that SR141716A acts as a competitive antagonist and reduces basal activity in the manner of an inverse agonist in these cells.

In some experiments, SR141716A has been found to be more potent in blocking the actions of CB1 agonists than in eliciting inverse responses by itself. For example, in their study which focused upon rat brain membrane and brain sections, Sim-Selley and co-workers [41] suggested that SR141716A may bind to two sites on the cannabinoid receptor, a high-affinity site at which it exerts its competitive antagonism and a lower-affinity site at which it exerts its inverse agonism.

1.4.3 CB2 Antagonists

The first CB2 antagonist, SR144528 (**7;** Fig. 4), has been reported by M. Rinaldi-Carmona and co-workers at Sanofi Recherche [42]. SR144528 displays subnanomolar affinity for both the rat spleen and cloned human CB2 receptors ($K_i = 0.60 \pm 0.13$ nM). SR-144528 displays a 700-fold lower affinity for both the rat brain and cloned human CB1 receptors. JTE-907 has also been identified as an inverse agonist at CB2 [43]. CB2 receptor-transfected Chinese hamster cells exhibit high constitutive activity as well. This activity can be blocked by the CB2-selective ligand, SR144528, working as an inverse agonist [14]. AM630 has also been reported to be a CB2-selective antagonist [44].

2 Class A GPCR Structure

Ballesteros and Weinstein [45] have proposed a universal numbering scheme for Class A GPCRs. In this numbering system, the most highly conserved residue in each transmembrane helix (TMH) is assigned a locant of 0.50. This number is preceded by the TMH number and followed in parentheses by the sequence number. All other residues in a TMH are numbered relative to this residue. In this numbering system, for example, the most highly conserved residue in TMH2 of bovine rhodopsin is D2.50(83). The residue that immediately

precedes it is A2.49(82). The Ballesteros–Weinstein numbering system will be used throughout this chapter.

As revealed by the crystal structure of bovine rhodopsin at 2.8 Å [46], 2.65 Å [47], 2.6 Å [48], and 2.2 Å resolution [49], the general topology of a GPCR includes:

1. an extracellular N terminus;
2. seven transmembrane (TM) alpha helices arranged to form a closed bundle;
3. loops connecting TM helices that extend intra- and extracellularly; and,
4. an intracellular C terminus that begins with a short helical segment (Helix 8) oriented parallel to the membrane surface.

Ligand binding in GPCRs is thought to occur within the binding site crevice formed by the TMH bundle, to extracellular loops or to a combination of extracellular loop and binding site crevice residues. Agonists are thought to bind and produce a conformational change which initiates coupling to the G protein which is located inside the cell. An agonist-bound receptor activates an appropriate G protein that promotes dissociation of guanosine diphosphate (GDP). Although the interactions between receptor and G protein are poorly understood, both mutagenesis and biochemical experiments with a variety of GPCRs suggest that, first, ligand-induced receptor activation causes a change in the relative orientations of TMHs 3 and 6. This modification then affects the conformation of the G protein-interacting intracellular loops of the receptor and thus uncovers previously masked G protein-binding sites [50]. Second, as GDP is buried within the G protein, the receptor–G protein interaction must, in addition, promote changes in interdomain interactions [51]. Thus, to activate the G protein, the receptor has to deliver *two pieces of information*: (1) for the formation of the receptor–G protein complex and (2) to induce the exchange of bound GDP for guanosine triphosphate (GTP) on the heterotrimeric G protein, resulting in the dissociation of the G protein into active Gα-GTP and Gβγ subunits. Both the Gα-GTP and Gβγ subunits then interact with effectors to transduce the signal initiated by agonist binding.

2.1 The Class A GPCR-Activated (R*) State

The crystal structure of rhodopsin represents one state, the inactive state, conformation of the receptor [46–48]. While current research efforts are directed toward obtaining a structure of the rhodopsin-activated state, (Meta II), [52–54] no crystal or electron micrograph (EM) structure of rhodopsin in its fully activated Meta II state has been published. Yet, knowledge of the structure of this state is critical for modeling studies. Pharmacological studies show that agonists, antagonists, and inverse agonists do not bind to a single receptor conformation [55]. Many models of GPCRs fail to distinguish this fact, opting to dock agonists, antagonists, and inverse agonists in the same receptor model, usually based on the rhodopsin (inactive state) crystal structure. In contrast,

pharmacological, biophysical, and structural data, and data on constitutively active receptors all demonstrate that GPCRs exist in distinguishable conformations and, hence, *a single model for any receptor can never be adequate*. For this reason, models of the activated forms of GPCRs have become increasingly necessary for the development of a clear understanding of signal propagation into the cell. Experimental evidence points to a multiplicity of conformations related to the activation of the receptor, rendered important physiologically by the suggestion that different conformations may be responsible for coupling to different signaling pathways [56].

At present our primary structural information about the GPCR activation process comes from biophysical studies of rhodopsin (Rho) and the β2-adrenergic receptor (β2-AR). These studies have indicated that rotation of TMHs 3 and 6, as well as a conformational change in TMH6 (straightening in the CWXP hinge region of TMH6) occurs upon GPCR activation [57–62]. Recent in situ disulfide cross-linking studies [63] have confirmed an agonist induced rotation of the cytoplasmic end of TMH6 in the M_3 muscarinic receptor. At their intracellular ends, TMHs 3 and 6 in rhodopsin are constrained by a E3.49(134)/R3.50(135)/ E6.30(247) salt bridge that limits the relative mobility of the cytoplasmic ends of TMH3 and TMH6 in the inactive state [46] and acts like an *ionic lock* [64, 65]. During activation, P6.50 of the highly conserved CWXP motif in TMH6 of GPCRs may act as a flexible hinge, thus permitting TMH6 to straighten upon activation and moving its intracellular end away from TMH3 and upward toward the lipid bilayer [61]. A recent crystal structure of a photoactivated, deprotonated intermediate of Rho indicates that changes that accompany photoactivation are smaller than those predicted by biophysical studies [54]. It is unclear at this point the extent to which crystal-packing forces may have limited conformational change in this structure, and work is needed to determine the relationship of this activated form of Rho in crystals to the full-activated state, Meta II. For this reason, current models of the GPCR-activated state rely on biophysical data.

It would seem that one way to approach obtaining a model of the activated state would be via molecular dynamics (MD) simulations that start with the agonist bound in the inactive state. The timescale for activation has been estimated to be milliseconds for light activation of rhodopsin, [66] but seconds for activation of the β2-AR by its diffusible ligand [58]. It is not possible at the present time to perform MD simulations to study agonist-induced changes to the inactive (R) state to generate the activated (R*) state due to such long timescales for GPCR activation compared to the typical length of MD simulations (10–100 ns).

An alternate way to build activated state models is to create a model that reflects the conformational changes suggested to occur during GPCR activation as deduced from biophysical studies. Such model creation built on the biophysical literature has recently been described [56]. In Sect. 3, creation and refinement of homology models of the cannabinoid CB1 and CB2 inactive states, as well as creation of activated state models is described. These models

will be used to provide a molecular-level explanation for inverse agonism, neutral antagonism, and agonism at the CB1 and CB2 receptors (Sect. 4).

3 Models of the Cannabinoid CB1 and CB2 Receptors

3.1 Homology Models of the CB1/CB2 Inactive (R) State

Because rhodopsin has been the only GPCR crystallized to date, the most common way to build a model of another Class A GPCR (inactive state) is by homology modeling based on the crystal structure of bovine rhodopsin [46–49]. Such homology models have been built by several research groups for CB1 and CB2. Sequence alignment with bovine rhodopsin is achieved by using the highly conserved residues/sequence motifs across Class A GPCRs. Using the Ballesteros–Weinstein numbering system, [45] these are N1.50 in TMH1, D2.50 in TMH2, (D/E)RY in TMH3, W4.50 in TMH4, P5.50 in TMH5, CWXP in TMH6, and NPXXY in TMH7. The cannabinoid CB1 and CB2 receptors lack the highly conserved proline at 5.50. As detailed in an early paper [67] the second-most highly conserved residue in TMH5 of most Class A GPCRs is a Tyr at position 5.58 in the C-terminal portion of TMH5. The CB1 and CB2 receptors have two Tyr's in common in this region (CB1: YLMFWIGVTSVLL LFIV**Y**A**Y**MYILWKA; CB2: YLLSWLLFIAFLFSGII**Y**T**Y**GHVLWKA). Bramblett and co-workers performed a manual *structural alignment* to show that Y294 in CB1 and Y210 in CB2 should be aligned with the highly conserved Tyr at 5.58 in other GPCRs. The Leu residue, eight residues back in the sequence, which normally is a Pro in Class A GPCRs, was assigned the locant 5.50 in order to preserve numbering system correspondence with other GPCRs [67]. Each of these highly conserved residues in each helix of CB1 and CB2 is shown in bold in Figs. 1 and 2.

3.1.1 Refining a Rhodopsin-Based Model: Sequence-Dictated Differences Between Rhodopsin and CB1/CB2

While CB1 and CB2 share most of the highly conserved sequence motifs present in the rhodopsin sequence (see above), rhodospin also has sequence motifs that dictate local structure that may be absent in CB1/CB2. Ballesteros and co-workers have proposed that the overall structures of rhodopsin and of aminergic receptors are very similar, although there are localized regions where the structure of these receptors may diverge. Furthermore, they proposed that several of the highly unusual structural features of rhodopsin are also present in aminergic GPCRs, despite the absence of amino acids that might have been thought critical to the adoption of these features. Thus, different amino acids or alternate microdomains can support similar deviations from regular α-helical structure, thereby resulting in similar tertiary structure. Such structural mimicry may be a

mechanism by which a common ancestor could diverge sufficiently to develop the selectivity necessary to interact with diverse signals, while still maintaining a similar overall fold. Through this process, the entire Class A family of receptors presumably shares the core function of signaling activation through a conformational change in the transmembrane segments that alters the conformation of the cytoplasmic surface and subsequent interaction with G proteins, despite their selectivity for a diverse group of ligands [68].

The majority of CB1/CB2 homology models have been built on the rhodopsin template [46] by first aligning the sequence of the human CB1 receptor [3] or CB2 receptor [11] with the sequence of bovine rhodopsin (Rho), mutating the residues in the Rho structure to the appropriate residues in CB1 or CB2, and then refining the resultant model through energy minimization or relaxation in molecular dynamics simulations [69–73].

The Reggio lab has taken additional steps to refine CB1 and CB2 models by studying the impact of sequence differences between CB1, CB2, and rhodopsin on the structures of CB1 and CB2. Changes to the general Rho structure that were necessitated by sequence divergences included the absence of helix kinking proline residues in TMH1 and TMH5, and five other important sequence-dictated divergences:

1. The difference in flexibility of the TMH6 CWXP motif due to the presence of G6.49 in CB1 versus F6.49 in CB2;
2. The difference in E-2 loop placement in CB1/CB2 versus rhodopsin;
3. The loss of the NPXXY$(X)_{5,6}$F motif in CB1/CB2;
4. The lack of a GG motif in TMH2 of CB1/CB2; and
5. The conformational effects exerted by S7.39 in CB1.

Each of these cases is discussed below.

TMH6 Differences Between CB1 and CB2

Even when the GPCR to be modeled has the highly conserved GPCR motifs, there still may be sequence-dictated variability from the rhodopsin structure. The CWXP motif in TMH6 is thought to act as a flexible hinge, permitting TMH6 to straighten during GPCR activation [74]. So can one expect that the flexibility and the helix geometry of TMH6 to be the same for all helices that have the CWXP motif? Both the CB1 and the CB2 receptors have the CWXP motif in TMH6. The sequence in CB1 is CWGP and in CB2, the sequence is CWFP. The Reggio lab undertook a Conformational Memories study of CB1 versus CB2 TMH6 in isolation to better understand the conformations possible for this important helix [75]. The Conformational Memories method employs multiple Monte Carlo/simulated annealing random walks and the Amber* force field. Conformational Memories has been shown to achieve complete sampling of conformational space of flexible molecules, to converge in a very practical number of steps, and to be capable of overcoming energy barriers efficiently [76]. The calculation is performed in two phases. In the first phase,

repeated runs of Monte Carlo/simulated annealing are carried out to map the entire conformational space of the helix. In the second phase, new Monte Carlo/simulated annealing runs are performed only in the populated regions identified in the first phase of the calculation. The final output is 100 structures at 310 K. Conformers are grouped using X-Cluster (Schrödinger, Portland, OR) according to their increasing root mean square (rms) deviation from the first structure output at 310 K. Because X-Cluster rearranges the conformers so that the rms deviation between nearest neighbors is minimized, any large jump in rms deviation is indicative of a large conformational change and hence identifies a new conformational family or cluster.

The Conformational Memories results for TMH6 in WT CB1 and CB2 indicated a dramatic difference in the range of conformations possible for each receptor subtype. For WT CB1, two major clusters were identified. One cluster exhibited a pronounced kink in the flexible hinge region, while the second cluster was straighter in the hinge region. For WT CB2, a single cluster was identified by Conformational Memories that had moderate kinking in the flexible hinge region. For all 100 conformers of WT CB1 generated by Conformational Memories, the average kink angle was $40.9° \pm 16.9°$ (standard deviation (SD)). For CB2, the average kink angle for all 100 structures was $24.6° \pm 4.3°$ (SD). The flexibility of the WT CB2 TMH6 s was very consistent with the flexibility reported for TMH6 in the β2-adrenergic receptor [74]. However, the results for WT CB1 suggested that CB1 TMH6 has increased flexibility. Barnett-Norris and co-workers hypothesized that this difference in flexibility may be due to the size of the residue that immediately precedes P6.50 (i.e., residue 6.49) in each receptor subtype. In the more flexible TMH6 (CB1), residue 6.49 is smaller in size—a glycine. In the less-flexible TMH6 (CB2), residue 6.49 is much larger in size—a phenylalanine. In order to test this hypothesis, Conformational Memories was used to compare the range of conformations possible for the *switch mutants*, CB1 G6.49F and CB2 F6.49G. For the CB1 G6.49F mutant, one population of moderately kinked helices with an average kink angle of $25.3° \pm 5.7°$ (SD) resulted. These results are similar to those for WT CB2. For the CB2 F6.49G mutant, two clusters resulted. The average kink angle for all 100 conformers was found to be $44.3° \pm 21.4°$ (SD). These results are similar to those obtained for WT CB1. Thus the extreme flexibility in the CWXP hinge region could be swapped between CB1 and CB2 simply by placing a Gly at residue position 6.49 (i.e., at the X of CWXP). Taken together, these results suggest that TMH6 in WT CB1 has been engineered to have greater flexibility and ability to bend due to the small Gly residue in the flexible hinge motif, CWGP, in CB1. Clearly, many of the helices output by Conformational Memories for WT CB1 are too bent to be able to be accommodated in the TMH bundle. Barnett-Norris and co-workers were able to use this output to choose an inactive state TMH6 for CB1 (Pro kink angle = 53.1°) and an activated state (see below) TMH6 (Pro kink angle = 21.8°). Another important outcome of the Conformational Memories studies was that while the wobble angle of CB2 TMH6 is similar to that in rhodopsin

(placing the extracellular end of TMH6 close to TMH5 in the context of the TMH bundle), the wobble angle of TMH6 in CB1 is quite different (pointing the extracellular end of TMH6 toward TMH3). As illustrated in the R model

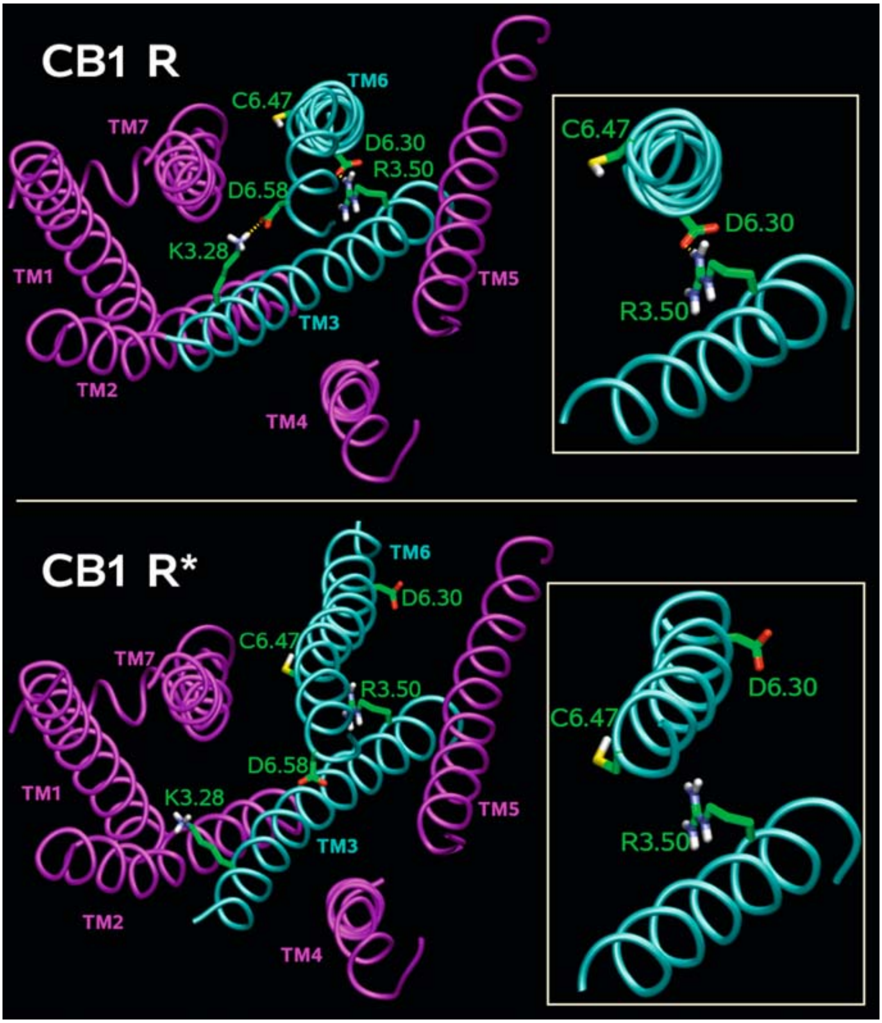


Fig. 5 (Top) An extracellular view of the CB1 transmembrane bundle model of the inactive (R) state is presented here. In the R state, the wobble angle of TMH6 causes the extracellular end to be close to TMH3. As a result, a salt bridge is possible between D6.58 and K3.28. (Inset) A salt bridge between R3.50 and D6.30 brings the intracellular ends of TMH3 and TMH6 close in the inactive state. (Bottom) An extracellular view of the CB1 transmembrane bundle model of the active (R*) state is presented here. In the R* state, TMH6 has straightened and both TMH3 and TMH6 have rotated counterclockwise. (Inset) At the intracellular end, the salt bridge between R3.50 and D6.30 has broken. (*See* Color Plate 4)

for CB1 in Fig. 5 below the flexibility of TMH6 and its different wobble angle enables a salt bridge to form between K3.28 and D6.58.

Extracellular Loop 2 in CB1/CB2 versus Rhodopsin

Despite the fact that the CB1 and CB2 receptors belong to the rhodopsin (Rho) family of G protein-coupled receptors (GPCRs), there are important differences between CB1/CB2 and Rho that impact the ligand-binding pocket in the TMH3-4-5-6 region. The CB1 and CB2 extracellular loop 2 (E-2 loops) are shorter than that of Rho (CB1 15 residues long; CB2 13 residues long; Rho 25 residues long) and there is no corresponding Cys residue in TMH3 of CB1 or CB2 that would cause the E-2 loop to dip down into the binding site crevice as the E-2 disulfide bridge with Cys3.25(110) causes in Rho [46]. However, there is a Cys residue at the extracellular end of TMH4 and a Cys near the middle of the E-2 loop in the CB receptors. Mutation results of these residues (C174,C179) in CB2 suggest that a disulfide bridge between these two Cys residues may exist [77]. This loop has been modeled with an internal C4.66(257)–C264 disulfide bridge in CB1 based upon mutation results from the Farrens lab [78], for example, which show that these two cysteines are required for high-level expression and receptor function.

The different spatial requirements of the E-2 loop are important because this difference permits the extracellular end of TMH 6 to occupy a different position in the TMH bundle than is seen in Rho. As discussed above in Section "TMH6 Differences Between CB1 and CB2," the small size of residue 6.49 in CB1 (a Gly) results in pronounced flexibility of the CWXP flexible hinge motif in TMH6 [75]. In the CB1 inactive (R) state, this extra flexibility in TMH6 permits the extracellular end of TMH6 to bend toward TMH3, resulting in the formation of a salt bridge between D6.58 (near the extracellular end of TMH6) and K3.28 in TMH3. In contrast, in the CB1 activated (R*) state, the K3.28(192) and D6.58(366) salt bridge is broken (N-O distance = 16.8 Å) (see Fig. 5) [79]. As the result of this important difference between Rho and the CB receptors, the binding site crevice around TMHs3-4-5-6 is likely to be different with the E-2 loop occupying less volume in the upper part of the binding pocket than does the E-2 loop in Rho.

Absence of the NPXXY(X)$_{5,6}$F Motif in CB1/CB2

The crystal structure of bovine rhodopsin [46] reveals an intracellular α-helical extension of TMH7 (K311 to C323). This domain, referred to as helix 8 (Hx8), is positioned along the intracellular surface of TMH1 [46]. Photoactivation of rhodopsin has been associated with movement of Hx8 as detected in cysteine cross-linking studies and in nitroxide spin label studies [80]. The highly conserved NPXXY(X)$_{5,6}$F motif of rhodopsin has been proposed to provide structural constraints via the aromatic interaction of Y7.53(306) in the NPXXY motif of TMH7 with F7.60(313) in Hx8, which rearrange in response to

photo-isomerization [81]. This same interaction has been shown to be important for the switching of the 5HT-2C receptor among multiple active and inactive conformations [82]. Taken together, these studies suggest that the Hx8 domain plays a role in docking specific G protein subunits and in the transition between conformational states.

The CB1 receptor possesses a defined intracellular helical segment (Hx8 D7.59(403) to P7.69(413)), as predicted by Fourier transform analysis of the primary sequence of the CB1 receptor [67]. Significant helicity was demonstrated when the CB1 401–417 peptide was present in an anionic micelle environment (sodium dodecylsulfate or phosphatidic acid), but not in aqueous media [83]. Recent NMR and circular dichroism studies also revealed significant α-helical structure of synthetic peptides in dodecyl phosphocholine micelles [84, 85] or sodium dodecylsulfate [86]. However, the CB1 receptor differs from the NPXXY$(X)_{5,6}$F motif, as residue 7.60(404) is a Leu in CB1. Anavi-Goffer and co-workers explored the ramifications of this deviation from the highly conserved NPXXY$(X)_{5,6}$F motif by comparing the signal transduction capabilities of the CB1 wild type with those of an L7.60F mutation which mimics the rhodopsin motif, and an L7.60I mutation which mimics the homologous CB2 receptor sequence [87]. While agonist and antagonist binding were unaffected, the two mutant receptors differed from wild type in their ability to regulate G proteins in the [^{35}S]GTPγS-binding assay. The L7.60F receptor exhibited attenuated stimulation by agonists WIN-55,212-2 and CP55940 but not HU-210, whereas the L7.60I receptor exhibited impaired stimulation by all agonists tested as well as by the inverse agonist SR141716A. Coimmunoprecipitation studies revealed that both mutant receptors were associated with $G\alpha_{i1}$ or $G\alpha_{i2}$, but not with $G\alpha_{i3}$. The mutant receptors internalized more rapidly than WT CB1 receptors, but could equally sequester G proteins from somatostatin receptor. Both the maximal N-type Ca^{2+} current inhibition by WIN-55,212-2 and its time course were significantly reduced by both mutants. Reconstitution experiments with pertussis toxin-insensitive G proteins revealed loss of coupling to $G\alpha_{i3}$ but not $G\alpha_{0A}$ in the L7.60I mutant. Furthermore, reconstitution of the WT and mutant receptors with $G\alpha_{i3}$ but not $G\alpha_{0A}$-enhanced basal facilitation ratio, suggesting that $G\alpha_{i3}$ is responsible for CB1 tonic activity. Molecular dynamics simulations of WT CB1 receptor and each mutant in a 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) bilayer suggested that the packing of Hx8 is different in each. The hydrogen-bonding patterns along the helix backbones of each Hx8 also are different, as are the geometries of the elbow region of Hx8 (R7.56(400)-K7.58(402)). This study demonstrates that the evolutionary modification to NPXXY$(X)_{5,6}$L contributes to maximal activity of CB1 receptor, and provides a molecular basis for the differential coupling observed with chemically different cannabinoids. This study also shows that the change from the NPXXY$(X)_{5,6}$F motif to NPXXY$(X)_{5,6}$L or NPXXY$(X)_{5,6}$I (in CB2) alters the packing of Hx8 with the TMH bundle and that use of a Rho-based Hx8 conformation is not advisable in CB1/CB2 models [87].

Transmembrane Helix 2 in CB2

In their preliminary substituted cysteine accessibility method (SCAM) study of the CB2 receptor, the Song lab found that one native cysteine was primarily labeled by methanethiosulfonate (MTS) reagents, C2.59. These results indicated that this residue is accessible from within the binding site crevice [88]. The rate of reactivity with MTS suggested that this labeled Cys was likely in a helix/helix interface rather than facing directly into the binding pocket. In the Class A aminergic GPCRs, (with the exception of most of the muscarinic receptor family) there is a Pro at 2.59 in TMH2. In bovine rhodopsin, no Pro exists in TMH2, but TMH2 does have a GGXTT motif that begins at G2.57 [88] and ends at T2.61 [92]. This GGXTT motif causes a local distortion in the TMH2 helix backbone that may be mimicked by P2.59 in most of the aminergic GPCRs. In the CB1 and CB2 receptors, there is neither a GGXTT motif nor a P2.59. The original TMH2 modeled by the Reggio lab for CB2 had been modeled as a regular α-helix in which C2.59 faced lipid. Therefore, the Song SCAM results for TMH2, prompted another look at the modeled conformation of TMH2.

Both serines and threonines have been shown to be able to act as hinge residues to affect the conformation of an α-helix via an intrahelical hydrogen bond between the Oγ atom of the Ser or Thr (in a g^- or $+60^\circ$ $\chi 1$) and the i-3 or i-4 carbonyl oxygen of the helix backbone. This is of particular significance for membrane proteins [89]. S2.54(84) is a lipid-facing residue that is intracellular to the region of distortion in other Class A receptor TMH2 s. Using the biased Monte Carlo/simulated annealing method, Conformational Memories [76], the Reggio lab tested the hypothesis that S2.54(84) in a $g^-(+60^\circ)$ $\chi 1$ forms an intrahelical hydrogen bond that produces an alteration from normal α-helicity in CB2 TMH2. CM results indicated that S2.54(84) can indeed influence the backbone conformation of TMH2. This influence was extracellular to S2.54(84), allowing the highly conserved residue, D2.50, to remain oriented as in rhodopsin. While a statistically significant difference in TMH2 helix bend angles was not seen, a statistically significant difference in another measure of helix geometry, wobble angle, was seen. Conformational Memories calculations suggested that S2.54(84) serves to introduce a distortion from normal α-helicity in TMH2 by allowing a wobble in TMH2 that places C2.59 in the TMH2–TMH3 interface and accessible to MTS reagent. The corresponding residue in bovine rhodopsin, T2.59, faces lipid. This result suggests that there is a localized region in TMH2 (extracellular to S2.54) where the structure of CB2 and rhodopsin diverge.

Structural Effects of Serine 7.39 in CB1

S7.39(383) faces into the ligand-binding pocket in CB1. Mary Abood's lab recently studied the importance of this serine to ligand binding and activation at the CB1 receptor by stably expressing in HEK-293 cells recombinant human

CB1 receptors in which S7.39 was mutated to Ala (S7.39A) [90]. [^{3}H]WIN55,212-binding properties at S7.39A were comparable to the WT receptor. In contrast, the S7.39A mutant resulted in a total ablation of [^{3}H]CP55940 high-affinity binding. In addition, the binding affinity of the classical cannabinoid, HU-210 was drastically reduced (100-fold) at the S7.39A mutant. Similarly, the EC_{50} for GTPγS binding for HU-210-mediated activation of the S7.39A receptor was increased by >200-fold. These results clearly suggested that Ser7.39 plays a crucial role in mediating ligand-specific interactions for CP55940 and HU-210 at the hCB1 receptor.

The loss of high-affinity CP55940 binding at the S7.39(383)A mutant suggested the introduction of a steric block, one that cannot be caused by greater bulk of the mutated residue side chain in this case, as the amino acid change from a Ser to Ala is a size-conservative change. The Reggio lab tested the hypothesis that S7.39(383) in a g– $\chi1$ conformation may induce a helix bend in TMH7 that provides space for the binding of CP55940; and that mutation of S7.39(383) to Ala may alter this binding region space, precluding CP55940 binding.

Conformational Memories (CM) calculations revealed that the presence of a Ser at position 7.39 in WT CB1 TMH7 can induce a greater bend (15.1° in WT) compared with the S7.39(383)A mutant TMH7 (8.2°). Thus, when S7.39(383) (which is located on the interior face of TMH7) adopts a g– $\chi1$, it causes the top portion of the helix to bend away from S7.39(383). The net result is that the extracellular end of TMH7 pulls away from the TMH bundle. Replacement of S7.39(383) with an Ala results in the moderation of this kink (due to the lack of hydrogen-bonding capability of the Ala sidechain), bringing the extracellular end of TMH7 more into the TMH bundle. Results of this study permitted a refinement in the geometry of TMH7 in the CB1 R and R* models by incorporation of a TMH7 conformation selected from the CM study (Bend angle = 15.1°, Wobble = –164.6°, and FaceShift = 9.0°).

4 Inverse Agonism, Neutral Antagonism, and Agonism

The extended ternary complex model for GPCR activation invokes the existence of two receptor conformational states, a ground or inactive R state, and an active R* state, which are in equilibrium with each other [91]. An agonist has higher affinity for R* and agonist binding is thought to shift the equilibrium toward R*, resulting in G-protein activation with an increase in GDP/GTP exchange. An inverse agonist has higher affinity for R and its binding shifts the equilibrium toward R, thus suppressing ligand-independent (constitutive) activation [92]. The binding of a neutral/null antagonist is thought not to alter the equilibrium between R and R* because the neutral antagonist has equal affinity for both states.

It is generally assumed that a common molecular activation mechanism for Class A GPCRs exists. The essential components of this mechanism involving the

intracellular domains of TMH3 and TMH6, as well as helix rotations were discussed in Sect. 2.1 above [93]. However, this activation mechanism is also thought to involve a molecular switch within the ligand-binding pocket [94]. This switch is a noncovalent intramolecular interaction that exists within the ligand-binding pocket in the basal state of a GPCR and that must be disrupted to achieve an active state. A commonly proposed switch is the *rotamer toggle switch* which involves W6.48 on TMH6 [74, 94, 95]. Khorana and co-workers [96] have reported that even in the dark (inactive) state of rhodopsin, only some strong constraints exist, whereas the majority of the molecule experiences conformational flexibility. Light activation of rhodopsin, therefore, does not require the breaking and forming of thousands of specific contacts within nanoseconds. Instead, activation requires only a few specific contacts restricting the inactive state, including indole side chain contacts of tryptophan residues, to break on activation. These changes can then be transmitted through the entire membrane protein because of its dynamic plasticity. One of the tryptophan residues that Khorana and co-workers have reported to be restricted in rhodopsin is W6.48(265). In the dark (inactive) state of rhodopsin, the beta-ionone ring of 11-cis-retinal is close to W6.48(265) of the CWXP motif on TMH6 and acts as a lynch pin constraining W6.48 in a χ_1 = g+ conformation [46–48]. In the light activated state, the beta-ionone ring moves away from TMH6 and toward TMH4 where it resides close to A4.58(169) [97]. This movement releases the constraint on W6.48(265) making it possible for W6.48(265) to undergo a conformational change. Lin and Sakmar [59] reported that perturbations in the environment of W6.48(265) of rhodopsin occur during the conformational change concomitant with receptor activation. This suggests that the conformation of W6.48(265) when rhodopsin is in its inactive/ground state (R; χ_1 = g+) changes during activation (i.e., W6.48(265) χ_1 g+ $\rightarrow$ trans) [74]. In the Class A aminergic receptors, a highly conserved cluster of aromatic amino acids is found on TMH6 that face the binding site crevice bracketing W6.48 (F6.44, W6.48, F6.51, and F6.52) [74]. Shi and co-workers used mutation studies of the β2-adrenergic receptor combined with the biased Monte Carlo technique of CM to propose that a C6.47 χ1 *trans*/W6.48 χ1 *g*+/F6.52 χ1 *g*+ $\rightarrow$ C6.47 χ1 *g*+/W6.48 χ1 *trans*/F6.52 χ1 *trans* transition is the key switch within the binding site crevice of the β2-adrenergic receptor that leads to the R* state of the β2-AR [74].

4.1 The F3.36/W6.48 Rotamer Toggle Switch at the CB1 Receptor

Restriction of W6.48 by a TMH6 aromatic cluster is not possible in the cannabinoid receptors as the CB1 receptor has leucines at 6.44, 6.51, and 6.52. Instead, the CB1 receptor contains a microdomain of aromatic residues that face into the ligand-binding pocket in the TM3-4-5-6 region, including F3.25(189), F3.36(200), W4.64(255), Y5.39(275), W5.43(279), and W6.48(356). Singh and co-workers used the biased Monte Carlo/simulated annealing

technique of CM combined with receptor modeling to suggest that the F3.36(200)/W6.48(356) interaction may act as a mimic of the 11-cis-retinal/ W6.48 interaction in the rhodopsin dark state and may serve as the toggle switch for CB1 activation, with F3.36(200) $\chi 1$ *trans*/W6.48(356) $\chi 1$ *g*+ representing the inactive (R) and F3.36(200) $\chi 1$ *g*+/W6.48(356) $\chi 1$ *trans* representing the active (R*) state of CB1 [98]. Figure 6 illustrates this with F3.36 and W6.48 engaged in a direct aromatic stack in the R state and rotated away from each

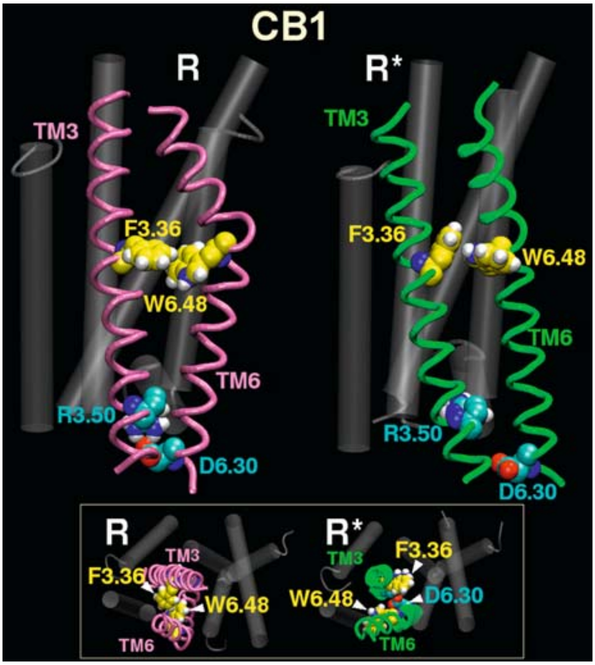


Fig. 6 The relationship between F3.36(200) and W6.48(356) in the inactive (R) and active (R*) states of CB1 as predicted by molecular modeling is illustrated here. The major view is from TMH5 looking toward TMHs3/6. *Left,* in the R state, W6.48(356) adopts a g+ $\chi 1$, whereas F3.36(200) adopts a trans $\chi 1$. In this arrangement, W6.48(356) and F3.36(200) are engaged in an aromatic-stacking interaction that stabilizes the R state. By analogy with Rho, the CB1-inactive state is also characterized by a salt bridge between R3.50(214) and D6.30(338) at the intracellular side of CB1 that keeps the intracellular ends of TMH3 and 6 close. The TMH6 kink extracellular to W6.48(356) permits a hypothesized salt bridge between K3.28(192) and D6.58(366) to form [51]. This salt bridge is made possible by the profound flexibility in TMH6 due to the presence of G6.49(357) in the CWXP motif of TMH6 [25]. *Right,* in the R* state, W6.48(356) and F3.36(200) have moved apart due to rotation of TMH3 and -6 during activation. W6.48(356) has adopted a trans $\chi 1$ and has moved toward the viewer and F3.36(200) has adopted a g+ $\chi 1$ and has moved away from the viewer. The R3.50(214)/D6.30(338) salt bridge is broken and the proline kink in TMH6 has moderated. *Inset,* this inset provides an extracellular view of CB1. Here it is clear that in R, F3.36(200) and W6.48(356) are engaged in an aromatic stacking interaction, but in R*, F3.36(200) and W6.48(356) are no longer close enough to interact [95]. (*See* Color Plate 5)

other in the R* state. A detailed functional analysis of mouse CB1 F3.36A and W6.48A mutants undertaken to test this toggle switch hypothesis showed statistically significant increases in ligand-independent stimulation of GTPγS binding for a F3.36A mutant versus. WT mCB1, while basal levels for the W6.48A mutant were not statistically different from WT mCB1. These results suggested that F3.36 may function as a *linch pin* restraining W6.48 from moving to an active state conformation in the CB1 receptor [95].

The identification of the rotamer toggle switch is the key to the rational design of inverse agonists versus agonists. For the cannabinoid receptors, this means that ligands that preferentially stabilize the F3.36(200) $\chi 1$ *trans* / W6.48(356) $\chi 1$ $g+$ interaction in CB1 will function as inverse agonists, while ligands that preferentially promote/stabilize the F3.36(200) $\chi 1$ $g+$/W6.48(356) $\chi 1$ *trans* conformation will function as agonists at CB1. Ligands that show equal preference for these two states will function as neutral antagonists. In the following section, we first examine the binding of cannabinoid inverse agonists and neutral antagonists at CB1. Readers are referred to another chapter in this volume by Yanan Zhang, Herbert H. Seltzman, Marcus Brackeen, and Brian Thomas, entitled "Structure-Activity Relationships and Conformational Freedom of CB1 Receptor Antagonists and Inverse Agonists" that approaches this topic from a QSAR perspective.

4.2 Cannabinoid Inverse Agonists/Neutral Antagonists

In SCG neurons expressing WT CB1, SR141716A (**7**; Fig. 4)) competitively antagonizes the Ca^{2+} current effect of the cannabinoid agonist, WIN55212-2 (**3;** Fig. 3), and behaves as an inverse agonist by producing opposite current effects when applied alone. In contrast, in neurons expressing CB1 with a K→A mutation at residue 3.28(192) (i.e., K3.28A), SR141716A competitively antagonizes the effects of WIN55212-2, but behaves as a neutral antagonist by producing no current effects itself [6].

One of the significant features of the CB1 R TMH bundle is a salt bridge between K3.28(192) and D6.58(366) (see Fig. 5 and Section "TMH6 Differences Between CB1 and CB2" above). Unlike the intracellular R3.50/E6.30 (or R3.50/D6.30) salt bridge shown to stabilize GPCRs in their inactive states [64], the extracellular K3.28/D6.58 salt bridge in CB1 (present only in the inactive state of CB1) appears to be important for positioning K3.28 for ligand interaction in the inactive state, rather than for stabilizing the receptor in the inactive state [99].

The CB1 TMH3-4-5-6 region is rich in aromatic residues that face into the ligand-binding pocket, including F3.25(189), F3.36(200), W4.64(255), Y5.39(275), W5.43(279), and W6.48(356). The CB1 inverse agonist SR141716A is a highly aromatic compound. Hurst and co-workers hypothesized that aromatic-stacking interactions might be important for the binding of SR141716A at CB1 [99]. Shire and co-workers [77] have shown in CB1/CB2

chimera studies that the TMH4-E-2-TMH5 region of CB1 contains residues critical for the binding of SR141716A. In Monte Carlo/Stochastic Dynamics Simulations of the inactive state of WT CB1, McAllister and co-workers [100] found a persistent aromatic stack between Y5.39(275) and W4.64(255) that appeared to be important for stabilizing the positions of TMHs4 and 5 in the TMH bundle on the extracellular side and a second aromatic stack between F3.36(200), W5.43(279), and W6.48(356) that appeared to be open for additional interaction with ligand. Hurst and co-workers, therefore, pursued the aromatic residue-rich TMH3-4-5-6 region as the binding site for SR141716A [99]. Additional mutation studies confirmed that F3.36(200), W5.43(279), and W6.48(356) were part of the SR141716A-binding pocket [101]. In the Hurst and co-workers' model of the CB1 R bundle, the TMH3-4-5-6 region is characterized by a W6.48(356)/W5.43(279)/F3.36(200) and a Y5.39(275)/

W4.64(255)/F5.42(278) aromatic cluster [99]. In the R* TMH bundle, an aromatic cluster exists between W6.48(356)/W5.43(279)/Y5.39(275)/W4.64(255)/F5.42(278). Modeling studies indicated that SR141716A can insert itself into this aromatic residue-rich region to become an integral part of an *extended aromatic cluster in both the R and R* states of CB1*. This docked position is consistent with mutation studies at CB1 C7.42(386) conducted in the Farrens' lab which showed that enlargement of residue 7.42 interferes with SR141716A binding [78]. However, the model indicated that there was one key interaction that was available to SR141716A only in the inactive state, a hydrogen bond between the carboxamide group and K3.28 [99].

To test if the interaction between SR141716A and K3.28 was the key to its inverse agonism, Hurst and co-workers constructed a *mutant thermodynamic cycle* that combined the evaluation of SR141716A affinity at WT CB1 and K3.28A with an evaluation of the WT CB1 and K3.28A affinities of a *mutant SR141716A analog*, VCHSR (**9;** Fig. 4) that lacks hydrogen-bonding potential at C-3 [99]. For SR141716A in cloned human, WT CB1, K_d was 2.3 ± 1.1 nM, while this value was 39.6 ± 10.5 nM for SR141716A at CB1 K3.28(192)A, suggesting that this ligand is involved in a strong interaction with K3.28 in WT CB1. The K_i value for VCHSR binding in cloned human WT CB1 and CB1 K3.28(192)A cell lines verus [^{3}H]SR141716A was 31.3 ± 9.6 nM and 35.2 ± 1.4 nM indicating that K3.28 is not an interaction site for VCHSR. These binding affinities were used in a thermodynamic cycle calculation which indicated that a direct interaction likely occurs between the C-3 substituent of SR141716A and K3.28 in WT CB1 [99].

Because the C-3 substituent in VCHSR lacks hydrogen-bonding potential, it cannot form a hydrogen-bonding interaction with K3.28(192) despite its proximity to the K3.28(192)-D6.58(366) salt bridge. However, VCHSR can engage in similar aromatic stacking interaction as SR141716A in the CB1 R and R* states [99]. As a result, VCHSR should have nearly equal affinity for both the R and R* states causing it to behave as a neutral antagonist. Consistent with this prediction, in SCG neurons VCHSR at 1 μM and at 10 μM could antagonize the effect of WIN in inhibiting Ca^{2+} currents, but when applied alone VCHSR

produced an effect that was not statistically different from vehicle. Thus, the experimental results support the hypothesis that the interaction between K3.28(192) and SR141716A in WT CB1, an interaction that is possible only in the R state, causes SR141716A to have higher affinity for the R state and consequently behave as an inverse agonist [99].

In subsequent studies, Hurst and co-workers designed, synthesized, and evaluated a new set of SR141716A analogs in which modifications only to the C-3 substituent of SR141716A were made that still preserved the geometry of this substituent in SR141716A [79]. The work was designed to test two hypotheses:

1. It is the carboxamide oxygen of the C-3 substituent of SR141716A that interacts directly with K3.28(192); and
2. Interaction with K3.28(192) is crucial for the production of inverse agonism for biarylpyrazoles, such as SR141716A.

The absence of the piperidine nitrogen was not found to affect affinity, whereas the absence of the carboxamide oxygen resulted in a reduction in affinity. CB1-docking studies in an inactive state model of CB1 resulted in a trend for ligand/CB1-interaction energies that was consistent with the trend in WT CB1 Ki's versus [^{3}H]CP55940**.** In calcium channel assays, all analogs with carboxamide oxygens (which can hydrogen bond to K3.28) were found to be inverse agonists, while those that lacked this group (and therefore were unable to hydrogen bond with K3.28) were found to be neutral antagonists. Taken together, these results support the hypothesis that it is the carboxamide oxygen of the C-3 substituent of SR141716A that engages in a hydrogen bond with K3.28(192) in WT CB1. Further, functional results support the hypothesis that the interaction of SR141716A with K3.28(192) may be key to its inverse agonism.

4.2.1 Inverse Agonist Docking Relationship to Toggle Switch

Figure 5 shows that K3.28 is only available to the aromatic microdomain TMH4-5-6 region of CB1 in the inactive state (R). Docking studies show that when SR141716A docks in the R state to take advantage of the placement of K3.28, its dichlorophenyl ring *sits on top* of F3.36 and forms an aromatic stacking interaction with this residue (F3.36 in turn stacks with W6.48). In this docking position, SR141716A would block any movement of the F3.36/W6.48 toggle switch [101].

4.3 Agonism at Cannabinoid CB1/CB2 Receptors

Today, it is becoming increasingly recognized that all agonists do not promote the same GPCR R* conformation [102]. The idea that a receptor can adopt more

than one activated R* state is based upon the concept of agonist-directed trafficking of a receptor stimulus. This concept has been used to explain the ability of structurally diverse agonists to activate different G-protein-mediated signaling [103]. According to this model, each agonist is theoretically able to promote its own specific active receptor state, leading to a limitless number of receptor conformations, Rn*. The idea that agonist ligands may *imprint* a particular but subtlely different R* conformation on the receptor appears to be true in the cannabinoid field where early studies have shown selective G-protein coupling triggered by structurally different agonists [104, 105]. The reader is referred to another chapter in this volume by A. C. Howlett, L. W. Padgett, and J.-Y. Shim entitled, "Cannabinoid Agonist and Inverse Agonist Regulation of G-Protein Coupling" for a complete discussion of this important topic.

The idea that structurally diverse cannabinoid agonists may induce unique R* conformations is consistent with the cannabinoid mutation and modeling literature. This literature has clearly shown that the four structural classes of cannabinoid agonists each interact with unique sets of amino acid residues. These sets may have some specific residues in common, but all sets have some geometrical overlap such that ligands from one structural class can displace ligands from other structural classes. In the section below, we discuss the binding sites identified for each ligand structural class through a combination of mutation and modeling studies. The reader is referred to another chapter in this volume by Mary Abood entitled, "Molecular Biology of Cannabinoid Receptors: Mutational Analyses of the CB receptors" for a more complete review of the cannabinoid mutation literature.

4.3.1 Aminoalkylindole-Agonist Binding

The first reported mutation of the CB1 receptor indicated that the binding site of the aminoalkylindoles (AAIs) diverged from those of the other three classes of cannabinoid agonists. In 1996, Song and Bonner [106] reported that mutation of K3.28 in CB1 to Ala resulted in a complete loss of HU-210, CP55940, and anandamide (but not WIN55212-2) binding and >100-fold decrease in EC_{50} for HU-210, CP55940, and anandamide (but not WIN55212-2) upon this mutation. These results were confirmed later by Chin and co-workers [107].

Two additional mutation studies began to shed light upon where the AAIs may bind at CB1 and CB2. WIN55212-2 exhibits a 15- to 20-fold higher affinity for the CB2 receptor than for the CB1 receptor. Mutation studies performed by Song and co-workers showed that this selectivity could be lost at CB2 with a F5.46V mutation and switched in favor of the CB2 receptor with a CB1 V5.46F mutation [108]. In CB1/CB2 chimera studies, Shire and co-workers showed that the TMH4-E2-TMH5 region of CB1 contains residues critical for the binding of SR141716A and WIN55212-2 [77].

The importance of aromaticity for proper AAI interaction with CB1 has been illustrated by Huffman and co-workers [109]. These investigators reported that replacement of the naphthyl ring of WIN55212-2 with an alkyl (CH_3) or

alkenyl ($(CH_3)_2C=CH$) group resulted in complete loss of CB1 affinity (Ki > 10,000 nM) in both cases. Eissenstat and co-workers [110] reported that replacement of the naphthyl ring of WIN55212-2 with a hydrogen resulted in a compound with a very high IC_{50} at CB1. These findings are significant because they underscore that an aromatic system as part of the C-3 substituent of WIN55212-2 may be important and because it suggests that the requirement at this position is not simply a requirement for any hydrophobic moiety.

Modeling studies suggested that due to the highly aromatic character of WIN55212-2, this ligand (like SR141716A) would likely bind within the TMH3-4-5-6 aromatic microdomain [101]. Modeling studies suggested that aromatic residues in the TMH3-4-5-6 region form a network or cluster with which WIN55212-2 interacts. At its binding site in CB1 R*, WIN55212-2 was found to have a direct aromatic stacking interaction with W5.43(279) and W6.48(356) and two direct-stacking interactions with F3.36(200). In binding, WIN55212-2 becomes part of an aromatic cluster that includes F3.36(200)/W6.48(356)/W5.43(279)/Y5.39(275)/W4.64(255) in the minimized complex. No hydrogen-bonding interactions were identified for WIN55212-2 in CB1 R* [101]. The carbonyl oxygen of WIN55212-2 would appear to be a likely hydrogen-bond acceptor; however, rigid AAI analogs in which the carbonyl group was replaced with a methylene group, the E naphthylidene indenes have been shown to retain high affinity at both the CB1 and CB2 receptors [111]. This result is consistent with reports by Huffman and co-workers that cannabimimetic indoles retain high CB1 affinity even when all hydrogen-bonding potential has been removed. (See compound *26* in [112].)

In their mutation study of the CB1 TMH3-4-5-6 aromatic microdomain, McAllister and co-workers found that the binding of WIN55212-2 was affected by the F3.36A, W5.43A, and W6.48A mutations, suggesting that these residues are part of the WIN55212-2-binding site. In contrast, the affinity of the cannabinoid agonist, CP55940 was unaffected by the F3.25A, F3.36A, W5.43A, or W6.48A mutations making CP55940 an appropriate choice as the radioligand for binding studies [101]. The CB1 R* model that emanated from the McAllister et al. studies has been used subsequently to study the SAR of 1-alkyl-3-(1-naphthoyl)indoles [113].

Shim and Howlett have proposed two different possible binding sites for the AAIs that are distinguished by the orientation of the AAI aroyl group within the binding pocket [114]. In the *aroyl-up* model, the ligand forms an aromatic cluster using residues in TMHs2-3. Aromatic–aromatic interactions occur between F2.61(174) and the WIN55212-2 naphthyl ring, and between F7.35(379) and the indole ring. F2.61(174) is also involved in aromatic–aromatic interactions with H2.65(178) and F2.64(177) that interact in turn with F3.25(189). The *aroyl-up1* binding conformation that involves TMHs2-3, however, is not consistent with the mutation literature discussed above which points to the TMH3-4-5-6 region as the binding region for AAIs such as WIN55212-2.

For the *aroyl-up2* conformation, Shim and Howlett reported that AAI interaction occurred with aromatic residues in the TMH4-5 region of CB1

[114]. An aromatic–aromatic interaction between Y5.39(275) with both the naphthyl ring and the indole ring of WIN55212-2 appeared to be important for receptor-ligand binding. Y5.39(275) was also involved in aromatic–aromatic interactions with F5.42(278) and W6.48(356). Thus, the *aroyl-up2* conformation formed an aromatic cluster that is more consistent with the mutation literature. It is important to note that Shim and Howlett's model for CB1 differs in the TMH5 region from the Reggio lab model, such that W5.43 is not available and F5.42 is available for ligand interaction in the Shim–Howlett model. Mutation results reported by McAllister and co-workers [101] suggest that W5.43 is a key interacting residue in the WIN55212-2-binding pocket, while no mutation results for F5.42 have been reported in the literature. In recent work, Shim and Howlett have proposed a steric trigger mechanism for activation of CB1 by WIN55-212-2 that involves aromatic–aromatic interactions [114].

Aminoalkylindole-Agonist Binding Relationship to Toggle Switch

Modeling studies suggest and mutation results confirm that WIN55212-2 interacts with F3.36, W5.43, and W6.48 at CB1 [101]. In the inactive (R) state of CB1, W6.48 is shielded from most direct interactions by its direct aromatic stack with toggle switch partner, F3.36, a residue that is extracellular to W6.48 (see Fig. 6, left). It is only in the activated state that F3.36 and W6.48 move away from each other and thereby both become available for direct interactions with ligand (see Fig. 6, inset). Therefore, WIN55212-2 acts as an agonist at CB1 because its binding site requires direct interactions with both F3.36 and W6.48 and therefore favors the conformation in which the toggle switch is broken.

4.3.2 Classical/NonClassical Cannabinoid Agonist Binding

While the AAIs bear no obvious structural similarities with the classical/nonclassical/endogenous cannabinoids, the nonclassical cannabinoids (CBs) clearly share many structural features with the classical cannabinoids, for example, a phenolic hydroxyl at C-1 (C2′) and alkyl side chain at C-3 (C-4′), as well as the ability to adopt the same orientation of the carbocyclic ring as that in classical CBs [30]. Prior to the discovery of the cannabinoid CB1 receptor, cannabinoid SARs were developed by those who hypothesized that at least some of the effects produced by cannabinoids may be receptor mediated. The early SAR that emerged has been reviewed comprehensively by Razdan [115] and by Makriyannis and Rapaka [116]. These reviews consider both classical and nonclassical cannabinoid compounds. The reader is referred to the chapter by Raj Razdan in this volume entitled, "Structure Activity Relationships (SAR) of Classical Cannabinoids" which discusses recent SAR work on both the classical and nonclassical cannabinoids.

Because there is structural/conformational similarity between the classical and nonclassical cannabinoids, [30, 117–120] unified pharmacophores developed for

these two classes have agreed with one another and have lead to a consensus pharmacophore that involves the existence of the following at the CB1 receptor:

1. A hydrophobic-binding pocket of limited depth into which the C-3 (C-4′) alkyl chain fits such that the chain is nearly perpendicular to the aromatic ring [117, 121, 122]. Analogs with side chains of less than five carbons have no affinity for CB1. Highest affinity is associated with the 1′,1′-dimethyl-heptyl side chain.
2. Hydrogen-bonding sites for the phenolic hydroxyl of Ring A (see Fig. 3, **1**), the northern aliphatic hydroxyl (NAH) group (Ring C; see Fig. 3, **2**) [106, 121–123] and the southern aliphatic hydroxyl (SAH) group (see Fig. 3; **2**) [124, 125].
3. An occluded region behind the methyl group of the carbocyclic ring (Ring C; see Fig. 3, **1**) occupied by residues of the receptor itself [30].
4. A large hydrophobic pocket that accommodates SAH hydrophobic analogues and a smaller hydrophilic pocket that accommodates the SAH group (see Fig. 3, **2**) [124, 125].

Deducing the binding sites for classical cannabinoids such as HU-210 and for nonclassical cannabinoids such as CP55940 has been more difficult because other than the K3.28A mutation reported by Song and co-workers, most reported mutations of CB1 have had little effect on the binding of these ligands.

CP55940 and Other NonClassical Agonists

Shim and co-workers have used a molecular-docking approach that combined Monte Carlo and molecular dynamics simulations to identify putative-binding conformations of nonclassical cannabinoid agonists, including AC-bicyclic CP47497 and CP55940 (**2**) and ACD-tricyclic CP55244 [69]. These investigators used an inactive state model of CB1 for these docking studies based upon the x-ray crystal structure of rhodopsin [46]. Ligand placement was based upon the assumption of a critical hydrogen bond between the (phenolic) A-ring OH and the side chain N of K3.28(192). Two alternative binding conformations were considered with a conformation in which the C-3 side chain pointed inside the receptor chosen as the binding site conformation for which the ligand could achieve more interactions. Key hydrogen bonds were identified between both K3.28(192) and E(258) and the A-ring OH (see Fig. 3, **2**), and between Q(261) and the C-ring C-12 hydroxypropyl. In subsequent simulations, Shim and Howlett have proposed that a conformational change in the C-3 side chain of CP55244 acts as a steric trigger to activate CB1 [126].

Mutation results reported by McAllister and co-workers have shown that the affinity of the cannabinoid agonist CP55940 is unaffected by the F3.25A, F3.36A, W5.43A, or W6.48A mutations [101]. These results suggest that unlike WIN55212-2, CP55940 does not bind in the TMH3-4-5-6 aromatic microdomain. As described above in Section “Structural Effects of Serine 7.39 in CB1,”

Kapur and co-workers recently reported that the CB1 S7.39A mutant resulted in a total ablation of [^{3}H]CP55940 high-affinity binding [90]. The total loss of high-affinity CP55940 binding did not point to loss of a hydrogen-bonding interaction because loss of a hydrogen bonding interaction between uncharged partners can be expected to result typically in a 10- to 15-fold diminishment of affinity. Instead, this loss suggested the introduction of a steric block, one that cannot be caused by greater bulk of the mutated residue side chain in this case, as the amino acid change from a Ser to Ala is a size conservative change. The Reggio lab tested the hypothesis that S7.39(383) in a g– $\chi 1$ conformation may induce a helix bend in TMH7 that provides space for the binding of CP55940; and that mutation of S7.39(383) to Ala may alter this binding region space, precluding CP55940 binding. Modeling studies showed that when S7.39(383) (which is located on the interior face of TMH7) adopts a g– $\chi 1$, it causes the top portion of the helix to bend away from S7.39(383) [90]. The net result is that the extracellular end of TMH7 pulls away from the TMH bundle. Replacement of S7.39(383) with an Ala results in the moderation of this kink (due to the lack of hydrogen bonding capability of the Ala sidechain) bringing the extracellular end of TMH7 more into the TMH bundle. These results led to refinement of CB1 R and R* models as described in Section "Structural Effects of Serine 7.39 in CB1." Since the effect of this mutation was not as severe on HU-210 binding, the mutation also suggested that the binding site of CP55940 was higher (more extracellular) to that of HU-210.

Modeling results suggested that while CP55940 and HU-210 bind in the TMH1-2-3-7 region of the CB1 WT binding pocket, CP55940 binds higher (more extracellular) in this pocket and is therefore greatly impacted by the inward movement of the extracellular end of TMH7 upon the S7.39(383)A mutation. At the CB1 WT binding site identified by modeling studies in the R* state of CB1 (with new conformation of TMH7 incorporated), CP55940 is oriented with all three hydroxyls on the same face of the molecule. This orientation of the hydroxyls is consistent with the binding site model for CP55940 proposed by Xie and co-workers based upon NMR solution spectra [118]. Modeling studies showed that each of these hydroxyls finds a hydrogen-bonding interaction in the CB1 WT R* bundle model [90]. When CP55940 was docked in the CB1 R* model in its global minimum energy conformation, the phenolic hydroxyl of CP55940 formed a hydrogen bond with K3.28(192). The northern aliphatic hydroxyl (NAH; see Fig. 3) formed a hydrogen bond with S1.39(123), while the southern aliphatic hydroxyl (SAH; see Fig. 3) of CP55940 formed two hydrogen bonds with K373 in the EC-3 loop. In the first hydrogen bond, K373 acts as the hydrogen bond donor to the SAH hydroxyl oxygen. In the second hydrogen bond, the SAH hydroxyl hydrogen donates a hydrogen bond to the backbone carbonyl oxygen of K373. In this docked position, the carbocyclic A ring of CP55940 (see Fig. 3) is close to TMH7 at the level of S7.39(383). The fact that one of the identified hydrogen bonds is with K3.28(192) is consistent with K3.28(192)A mutation data [106]. In this docked position of CP55940, the ring structure of CP55940 is at and above

(extracellular to) the level of S7.39(383). This docked position is consistent with mutation studies at C7.42(386) conducted in the Farrens' lab which showed that enlargement of residue 7.42 (a residue that is intracellular to S7.39(383) and to the CP55940 docking site) does not interfere with CP55940 binding [78].

HU-210 Binding

Kapur and co-workers docked HU-210 in the global minimum energy conformation for its fused-ring structure [30] in the TMH2-3-7 region of CB1 R* using K3.28(192) as its primary interaction site [106]. In the energy-minimized HU-210/CB1 R* complex, K3.28(192) acts as a hydrogen bond donor to the phenolic oxygen of HU-210. The northern aliphatic hydroxyl (NAH; see **8** in Fig. 4) of HU-210 hydrogen bonds with both S7.39(383) and S1.39(123). For the S7.39(383) hydrogen bond, the NAH hydroxyl group acts as the hydrogen bond donor to the serine oxygen. For the S1.39(123) hydrogen bond, the NAH hydroxyl oxygen acts as the hydrogen bond acceptor. The ligand was found to have its greatest pairwise interaction energy with S7.39(383) followed by S1.39(123) and K3.28(192). For these interactions, the coulombic energy dominates the overall pairwise energy of interaction. The ligand also has significant interactions with C7.42(386), L7.43(387), and F2.57(170). For the C7.42(386) and L7.43(387) interactions, the van der Waals energy dominates the overall energy of interaction. The F2.57(170) interaction has significant van der Waals and coulombic contributions and appears to have arisen from the interaction of phenolic ring hydrogens with the aromatic ring of F2.57(170). In addition to the profound effect the S7.39(383)A mutation has upon CP55940 binding, the mutation also has a deleterious effect on the binding pocket of HU-210, thus causing the loss of two hydrogen bonds for HU-210 [90].

Classical/NonClassical Cannabinoid Agonist-Binding Relationship to Toggle Switch

Mutation studies have suggested that K3.28(192) is the primary interaction site for nonclassical cannabinoids such as CP55940 and classical cannabinoids such as HU-210 at CB1 [106]. In Fig. 5, it is clear that K3.28 is accessible to the TMH2-3-6-7 region of CB1 only in the CB1 R* state. TMH3-4-5-6 aromatic microdomain mutations (F3.25A, F3.36A, W5.43A, and W6.48A) have shown no effect on CP55940 binding suggesting that CP55940 does not bind in the CB1 TMH3-4-5-6 region [101]. A TMH7 S7.39A mutation has deleterious effects on CP55940 and HU-210 binding, but not on WIN55212-2 or SR141716A binding [90]. Taken together, these results suggest that CP55940 and HU-210 bind in the TMH2-3-6-7 region of CB1.

W6.48A mutation results suggest that W6.48 is not a direct interaction site for CP55940 [95] and it is clear in Fig. 5 that W6.48 is not available to the TMH2-3-6-7 pocket in CB1 R*. An isothiocyanate-labeled classical cannabinoid, AM841, selectively labels C6.47, [127] a residue accessible in the binding

pocket only in the R* state [60]. Thus it seems that CP55940 and HU-210(AM841) have no direct interaction with the F3.36/W6.48 toggle switch, but they do clearly prefer that state of CB1 that makes K3.28 available to the TMH2-3-6-7 pocket, that is, the CB1 R* state.

4.3.3 Endogenous Cannabinoid Agonist Binding

An analogy has been drawn in the literature between the C16-C20 portion of AEA (see **4**; Fig. 3) and the C-3 alkyl side chain of the classical cannabinoid, Δ^9-THC (see **1**; Fig. 3). Consistent with this hypothesis, replacement of the pentyl tail of AEA with a dimethylheptyl chain results in enhanced affinity (although not to the same degree as seen in the classical cannabinoids) [128, 129].

Endocannabinoid SAR indicates that the CB1 receptor recognizes ethanolamides whose fatty acid acyl chains have 20 or 22 carbons, with at least three homoallylic double bonds and saturation in at least the last five carbons of the acyl chain [130]. This is illustrated by the n-6 series of ethanolamide fatty acid acyl chain congeners, 22:4, n-6 ($K_i = 34.4 \pm 3.2$ nM), 20:4, n-6 ($K_i = 39.2 \pm 5.7$ nM), 20:3, n-6 ($K_i = 53.4 \pm 5.5$ nM); and 20:2, n-6 ($K_i > 1500$ nM) [131]. Endocannabinoid SAR also indicates that the CB1 receptor does not tolerate large endocannabinoid head groups; however, it does recognize both polar and nonpolar moieties in the head group region [130].

Biased Monte Carlo/simulated annealing (Conformational Memories) calculations of the following series: 22:4, n-6 ($K_i = 34.4 \pm 3.2$ nM), 20:4, n-6 ($K_i = 39.2 \pm 5.7$ nM), 20:3, n-6 ($K_i = 53.4 \pm 5.5$ nM); and 20:2, n-6 ($K_i > 1500$ nM) [131] have indicated that higher CB1 affinity is associated with endocannabinoids that can form tightly curved structures [132]. CB1 docking studies of the endogenous agonist anandamide in CB1 R* have suggested that anandamide binds in a U-shaped conformation in the TMH2-3-6-7 region using K3.28 as its primary interaction site [101]. In this interaction, K3.28 forms a hydrogen bond with the amide oxygen of anandamide. At the same time, the head group hydroxyl of anandamide is engaged in an intramolecular hydrogen bond with the amide oxygen. The formation of such an intramolecular hydrogen bond in anandamide helps the hydroxyl exist in a hydrophobic region and still satisfy a hydrogen bond. The residues that line the anandamide binding pocket are largely hydrophobic, including F2.57, F3.25, L3.29, V3.32, F6.60, F7.35, A7.36, Y6.57, S7.39 (hydrogen bonded back to its own backbone carbonyl oxygen), and L7.43. The interaction with F3.25 is a C-H$\cdots\pi$ interaction with the C5–C6 double bond in anandamide. In the R* bundle, F2.57 has an interaction with the amide oxygen. Here, an aromatic ring hydrogen interacts with one of the lone pairs of electrons of the amide oxygen. A similar interaction is evident in the x-ray structure of bovine rhodopsin [46] in which F5.38(203) points its positive edge into the backbone carbonyl oxygen of P4.60. The binding site conformation of anandamide is consistent with the x-ray crystal structure of anandamide's parent acid, arachidonic acid (20:4, n-6) complexed with adipocyte lipid-binding protein [133]. In this structure, arachidonic acid

clearly adopts a curved/U-shaped conformation similar to the bound conformation of anandamide reported by McAllister and co-workers.

The importance of K3.28 as a direct-interaction site for anandamide is supported by the work of Song and Bonner [106] who reported that anandamide was unable to compete for [^{3}H]WIN55212-2 binding in a human CB1 K3.28(192)A mutant and that the potency of anandamide in inhibiting cAMP accumulation was reduced >100-fold in this mutant. The CB1-binding site interactions identified for anandamide by McAllister and co-workers [101] agree with results first reported by Pinto and co-workers [134] which showed that the hydroxyl group in the head-group region of anandamide could be replaced by a methyl group without a loss in CB1 affinity. This result suggested that the hydroxyl group is *not essential* for anandamide binding and also that this hydroxyl may exist in a hydrophobic region of CB1. This result has been echoed in later endocannabinoid structure–activity relationship studies that showed, for example, that a cyclopropyl head group results in a very high CB1 affinity ligand [135]. In the binding site, identified for anandamide by McAllister and co-workers, the head group hydroxyl is located in a hydrophobic pocket and satisfies its hydrogen-bonding potential by forming an intramolecular hydrogen bond with the amide oxygen. This result is consistent with NMR solution studies of anandamide reported by Bonechi and co-workers [136] who found that this intramolecular hydrogen bond in anandamide persists in solution.

The binding site region identified for AEA by McAllister and co-workers has received support from modeling studies performed by Brizzi and co-workers [73]. These authors found that AEA docks in the TMH2-3-6-7 region of CB1 with the aliphatic chain directed toward the intracellular side of the receptor. The amide oxygen atom of AEA is hydrogen bonded to K3.28, while the hydroxyl group forms a hydrogen bond with S7.39. The n-pentyl tail of AEA is stabilized by lipophilic interactions with F3.25, V6.59, and F7.35 [73].

Endocannabinoid Entry Into CB1

It is important to mention that neither the CB1-binding site model proposed by Reggio and co-workers [101, 132] or by Brizzi [73] addresses one very important aspect of endocannabinoid acyl chain SAR, *why must the fatty acid chain be n-6?* Clearly in the CB1 binding sites described above, the unsaturated pentyl tail of AEA can contribute only van der Waal's interactions within the binding pocket While these are important interactions, they are not *all or nothing* interactions. Why then does shortening of the pentyl tail in AEA result in loss of ligand binding [130]? To address this question, the Reggio lab has hypothesized that this n-6 (pentyl tail) requirement originates not from the requirements of the final binding site itself but from requirements for endocannabinoid entry into the binding pocket from lipid via an initial interaction site on the lipid face of CB1.

It is commonly assumed that Class A GPCR ligands enter and exit the receptor via extracellular space. While this assumption makes sense for charged,

hydrophilic ligands such as the cationic neurotransmitters, a similar entrance/exit point is difficult to rationalize for hydrophobic ligands such as the endocannabinoids. Molecular dynamics simulations of anandamide (AEA) in a 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) bilayer have suggested that an elongated conformation of AEA is preferred in lipid with the last carbon of its arachidonyl chain having maximum probability at the bilayer center [137]. Ballesteros has proposed that β-branching residues (Val, Ile, or Thr) located (i,i + 3) or (i,i + 4) apart on an α-helix can form a hydrophobic groove into which an alkyl chain can fit [75]. Barnett-Norris and co-workers have previously shown that TMH6 in the CB receptors is rich in beta-branched amino acids (V, I, or T) and that in the CB1-inactive state, V6.43 and I6.46 form a groove on the *lipid face* of TMH6 close to the TMH6-7 interface into which an alkyl chain can fit [75]. NAMD2 molecular dynamics simulations of AEA in a model system composed of CB1 TMH6 in a DOPC bilayer showed that in the lipid bilayer, AEA can establish a high-energy interaction with TMH6 by first associating with the V6.43/I6.46 groove and then molding itself to the lipid face of TMH6 to establish a hydrogen-bonding interaction with the exposed backbone carbonyl of P6.50 [138].

The importance of the βXXβ groove to ligand recognition has been tested via CB1 V6.43A/I6.46A mutation and functional studies [139, 140]. CB1 V6.43A/I6.46A mutants were stably transfected into HEK293 cells. In competition-binding assays, SR141716A and WIN55212-2 retained their ligand-binding ability at the CB1 V6.43A/I6.46A mutant, whereas ligands with alkyl tails (HU-210, CP55940, and anandamide) failed to compete for specific [^{3}H]SR141716A binding. In competition with [^{3}H]CP55940, however, only ligands with alkyl tails (HU-210, CP55940, and anandamide) showed specific binding at the V6.43A/I6.46A mutant. The parallel mutation in CB2 resulted in essentially wild-type behavior. cAMP accumulation assays showed that both CB mutants remain functional with all ligands. These results suggest that the V6.43A/I6.46A mutation has separated the binding pockets of cannabinoid ligands with alkyl tails from those without alkyl tails and supports the hypothesis that the βXXβ groove may be part of a secondary (entry) binding site for certain CB ligands from the lipid bilayer into CB1.

The Maccarone lab has shown that an increase in membrane fluidity by treatment of rat C6 glioma cells with the lipid raft disruptor methyl-β-cyclodextrin (MCD) doubles the binding efficiency of AEA to the CB1 receptor [141], whereas a decrease in membrane fluidity by cholesterol enrichment has the opposite effect [142]. In contrast, MCD does not affect the binding of endocannabinoids to CB2 receptors, and subsequent G protein-dependent signaling through adenylate cyclase or MAP kinase [143]. Since an increase in membrane fluidity would aid (and decrease in fluidity would inhibit) lateral diffusion of lipid-derived molecules, such as AEA, these results are consistent with results reported above that suggest a lipid bilayer approach for endocannabinoids to CB1.

In a 45 ns NAMD2 molecular dynamics trajectory of the CB1 receptor in a POPC bilayer, the Caver program was used to identify a portal between TMH6 and TMH7 that connects the CB1-binding pocket at the level of W6.48 with the lipid bilayer [140]. The Reggio lab has hypothesized that once AEA is fished out of lipid and bound to the entry site, it may move through this portal to the interior of CB1. Experimental evidence that supports this hypothesis has come from covalent labeling studies performed by the Makriyannis lab [127]. Here, a classical cannabinoid ((–)-7′-isothiocyanato-11-hydroxy-1′,1′-dimethylheptylhexahydrocannabinol; AM841) that incorporates an isothiocyanate substituent at the end of the ligand's dimethylheptyl side chain was found to covalently label CB1. There are three TMH Cys residues that face into the binding site crevice of CB1:

1. C6.47(355), a residue located in the TMH6-7 interface in the R state and facing into the binding site crevice in the R* state; [60]
2. C7.38(382), a residue located in the TMH6-7 interface; and
3. C7.42(386), a residue that faces into the binding site crevice.

C6.47(355) is located intracellular to both C7.38(382), and C7.42(386) near the center of the TMH bundle. If AM841 entered the binding pocket from extracellular space, the first Cys residues that it would encounter would be those on TMH7. If, on the other hand, AM841 enters CB1 from lipid through the TMH6-TMH7 portal, C6.47 would be the first Cys residue that it would encounter. Experimental results from the Makriyannis lab have shown that AM841 covalently labels *only one Cys residue in CB1, C6.47(355)* [127].

Endogenous Cannabinoid Agonist-Binding Relationship to Toggle Switch

Barnett-Norris and co-workers have shown that interaction of a pentyl chain with the V6.43/I6.46 groove on CB1 TMH6 induces a moderation in the TMH6 proline kink angle (i.e., induces an activated state conformation for TMH6) and that this change correlates with a change in W6.48 $\chi 1$ from $g+ \rightarrow$ trans [75]. Thus the act of AEA binding to the proposed secondary (entry) binding site on the lipid face of CB1 may modulate the F3.36/W6.48 toggle switch such that AEA, when it enters the TMH binding site crevice, enters into an R* conformation of that receptor. Given the fact that the CB1 V6.43A/I6.46A mutation had similar effects on all ligands with tails (HU-210, CP55940, and AEA), [139, 140] it is possible that CP55940 and HU-210 may enter by a similar entry point and therefore modulate the toggle switch in a manner similar to that proposed here for AEA.

4.4 CB2 Receptor Models

The CB2 receptor has been less studied than the CB1 receptor and the toggle switch for CB2 activation has not yet been identified. The first model of the CB2 receptor, published by Song and co-workers, pre-dates the rhodopsin crystal

structure [46] and was a *de novo* model based upon available GPCR mutation data and the electron cryomicroscopy structure of bovine rhodopsin [144, 145]. Song and colleagues used this model to propose that WIN55212-2 binds at both the CB1 and CB2 receptors in the TMH4-5 region. F5.46 (which is V5.46 in CB1) was identified by these modeling studies as the primary residue responsible for the CB2 preference of WIN55212-2. CB1 V5.46F and CB2 F5.46 V swap mutation results showed that residue 5.46 was likely responsible for the 12- to 15-fold CB2 preference of WIN55212-2 [108].

Gouldson and co-workers [146] generated a CB2 receptor model from their previous model of the rat β2-adrenoceptor, which was based on the early electron cryomicroscopy structure of bovine rhodopsin [144]. In this model, the selective CB2 antagonist/inverse agonist, SR144528, penetrated deep into the TMH bundle in the TMH3-4-5 region. Docking studies revealed that SR144528 may have hydrogen-bonding interactions with both S4.53(161) and S4.57(165). Mutation of these two residues to Ala (S4.53(161)A and S4.57(165)A) resulted in a complete loss of SR144528 binding but retention of near-WT binding of WIN55212-2 and CP55940. Such a drastic effect upon SR144528 binding suggests that a steric problem has been created for SR144528 in each mutant. It is interesting that these are S→A mutations. By analogy to the CB1 S7.39A mutation discussed above [90] modeling of S4.53 and S4.56 in a g– $\chi 1$ may shed more light on the reason for this profound effect upon SR144528 binding.

In 2003, Xie and co-workers [70] published the first CB2 model based upon the rhodopsin crystal structure [46], which included loop regions. Residue side chains were positioned through a combination of rotamer library searches, and simulated annealing and minimization. Intermediate models of the 7TM helix bundles were analyzed in terms of helix tilt angles, hydrogen-bond networks, conserved residues and motifs, and possible disulfide bonds. The amphipathic cytoplasmic helix domain was also correlated with biological and site-directed mutagenesis data. Finally, the model receptor-binding cavity was characterized using solvent-accessible surface.

Salo and co-workers [71] built a rhodopsin homology model of the CB2 receptor to be used in virtual screening for new CB2 compounds. The CB2 model was utilized both in building database queries and in filtering hit compounds by a docking and scoring method. In G-protein activation assays, 1-isoquinolyl[3-(trifluoromethyl) phenyl]methanone (NRB 04079) was found to act as a selective agonist at the human CB2 receptor.

An *in silico* model of the hCB2 receptor was constructed by Yates and co-workers [72] to serve as a tool for designing new analogs of JWH-015. WIN55212-2 first was docked in the TMH4-5-6 region of a CB2 homology model based on the crystal structure of rhodopsin. WIN55212-2 was found to interact with F3.36(117), W5.43(194), and F5.46(197). The structure of JWH-015 was then superimposed on that of WIN55212-2 and the resulting dock was used for ligand design.

5 Allosteric Modulators of CB1

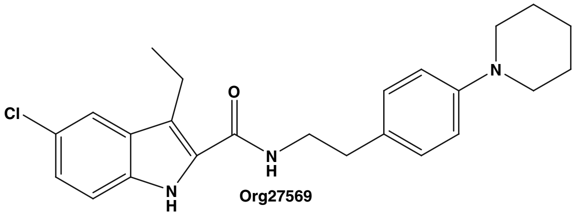
Tissue concentrations of endocannabinoids have been shown to increase in animal models of certain disorders such as multiple sclerosis and inflammatory pain. These increases in endocannabinoid levels often lead to a reduction in the severity of symptoms or to a slowing of disease progression [147]. Thus, one therapeutic avenue for the cannabinoid system would be to augment this apparently protective upregulation of the endocannabinoid system with ligands that either delay endocannabinoid degradation or that allosterically enhance endocannabinoid-induced activation of cannabinoid receptors.

Allosteric ligands are compounds that modulate the activity of GPCRs by interaction with accessory (allosteric) sites on these receptors. These sites are distinct from the agonist-binding site, which is termed the orthosteric site [148]. Occupation of allosteric sites on GPCRs has been shown to have an impact on orthosteric agonist affinity and/or efficacy [148]. Allosteric sites are topographically distinct from the orthosteric site; hence, the structural features that determine ligand binding to allosteric sites are different from those of orthosteric ligands. In contrast to the direct effects on receptor function that are mediated by orthosteric ligands, allosteric drugs may act by modulating receptor activity through conformational changes in the receptor that are transmitted from the allosteric to the orthosteric site and/or to effector-coupling sites [149].

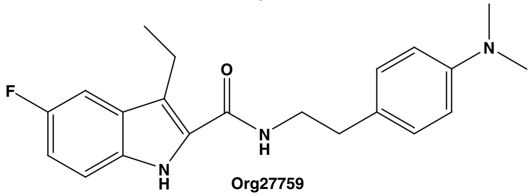
In 2005, Price and co-workers reported the first novel allosteric modulators of the cannabinoid CB1 receptor [150]. The compounds, Org 27569 (**10**; Fig. 7), Org 27759 (**11**; Fig. 7), and Org 29647 (**12**; Fig. 7) were synthesized at Organon, UK (www.organon.co.uk/research), and were found to display a number of characteristics commonly associated with allosteric modulators. Horswill and co-authors recently reported on another CB1 allosteric ligand, PSNCBAM-1 (**13**; Fig. 7) that produces effects similar to the Org compounds [151]. Both the Org compounds and the PSN compound display a ligand-dependent effect, whereby they enhance the specific binding of the CB1/CB2 receptor agonist [^{3}H]CP55940, inhibit the binding of the inverse agonist [^{3}H]SR141716A, and inhibit CP55940-stimulated [^{35}S]GTPγS binding. *Thus, these ligands are allosteric enhancers of agonist-binding affinity and allosteric inhibitors of agonist-signaling efficacy*. The Org compounds are neither agonists or antagonists in CB1 receptor assay systems ([^{35}S]GTPγS binding in brain membranes, mouse isolated vas deferens, and a CB1 cAMP reporter assay) they have no effect in the absence of agonist. While PSNCBAM-1 is inactive in yeast cells expressing constitutively active hCB1, in the [^{35}S]GTPγS-binding assay the PSN compound produced an inverse effect, albeit a significantly lower inverse effect than produced by SR141716A [151].

The first positive allosteric modulators of the CB1 receptor have recently been reported by Astra-Zeneca [152]. Twelve compounds have been identified that decrease the dissociation rate of [^{3}H]CP55940 from CB1 and increase AEA

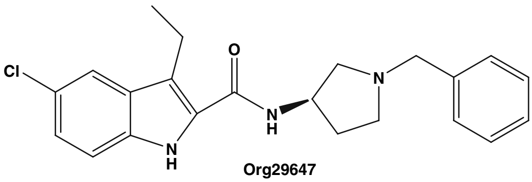
Fig. 7 Structures of cannabinoid CB1 allosteric modulators are illustrated here



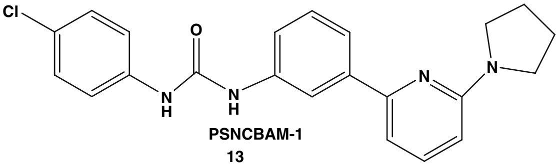
Org27569
10



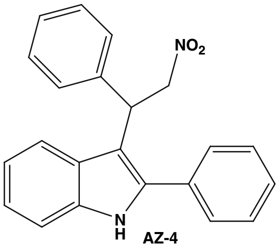
Org27759
11



Org29647
12



PSNCBAM-1
13



AZ-4
14

potency up to 5.4-fold. The structures of one of these, AZ-4 (**14**) is shown in Fig. 7.

In the muscarinic acetylcholine receptor field, it is generally accepted that prototypic druglike allosteric enhancers (e.g., gallamine and alcuronium) bind to an allosteric site that is located in the extracellular *funnel* leading to the

orthosteric monoamine-binding pocket and that these ligands may produce their effects by blocking the exit route of the orthosteric agonist. (See the excellent review by Schwartz and Holst [153] and references therein.) Inspection of the structures of the CB1 allosteric ligands **(10–14)** indicate that these ligands are highly aromatic and therefore a logical starting place for the identification of the binding sites for these ligands would be aromatic microdomains. But should this exploration be limited to the CB1 extracellular domains and TMH-binding site crevice or should the lipid-facing domains of the cannabinoid CB1 receptor also be considered given the high lipophilicity of reported CB1 allosteric modulators?

Barber and co-workers [154] have recently reported the use of a novel cavity-biased grand canonical Monte Carlo method (MMC) [155] to identify possible allosteric-binding sites at CB1 for the Org compounds (**10–12**). A model of CP55940 docked in the CB1 receptor R* state [90] was used for these calculations. The MMC method uses a novel fragment-driven approach that identifies regions of low-chemical potential for molecular fragments of the allosteric modulator. Overlap/proximity of these fragment regions consistent with the molecular structure of the modulator is used to identify possible binding sites. For example, for Org27569 (**10**; Fig. 7) these fragments would include acetamide (for the amide region of **10**), indole, benzene, and piperidine rings. The MMC method has been used successfully to identify potent and novel p38 kinase inhibitors [156] and to identify thermolysin and T4 lysozyme-binding sites [157]. Sites identified for Org27569 at CB1 included loop regions, lipid-facing sites, as well as a shallow position in the ligand-binding pocket Mutation studies are currently underway to help identify which of these sites is most likely the site of Org27569 binding at CB1.

Acknowledgments This work was supported by National Institutes of Health Grants: RO1 DA03934 and KO5 DA021358.

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Part IV
The Endocannabinoid System

Endocannabinoids as Modulators of Synaptic Signaling

Sachin Patel and Cecilia J. Hillard

Abstract One of the major roles of the CB1 cannabinoid receptor in the brain is to mediate short-term and some forms of long-term retrograde inhibition of synaptic transmission. The CB1 cannabinoid receptor is highly expressed in many brain regions. In particular, the CB1 receptor is localized to axon terminals of neurons that release either glutamate or GABA. The purpose of this review is to present and discuss the experimental evidence that presynaptic CB1 receptors function to inhibit neurotransmitter release. Furthermore, evidence is summarized that these CB1 receptors are innervated by endogenous cannabinoid ligands (endocannabinoids) that are mobilized from neurons postsynaptic to those terminals expressing the receptors. The triggers for endocannabinoid mobilization are discussed and the possible physiological roles of endocannabinoid-mediated changes in synaptic activity are presented.

Keywords Depolarization-induced suppression of inhibition · Long-term depression · Cannabinoid receptor · Retrograde inhibition · Hippocampus

Abbreviations

eCBs	Endocannabinoids
AEA	*N*-arachidonylethanolamine
2-AG	2-arachidonoylglycerol
CB1R	CB1 Cannabinoid Receptors
LTP	Long-term Potentiation
DSI	Depolarization-induced Suppression of Inhibition
CCK	Cholecystokinin
GABA	Gamma Aminobutryic Acid

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_10,

CB1R-ir	CB1R Immunoreactive
PV	Parvalbumin
eIPSPs	Stimulation-evoked, Inhibitory Postsynaptic Potentials
sIPSPs	Spontaneously Occurring, Action Potential-dependent Inhibitory Postsynaptic Potentials
mIPSPs	Miniature Inhibitory Postsynaptic Potentials
VDCC	Voltage-dependent Calcium Channels
PPR	Paired-pulse Ratio
FAAH	Fatty Acid Amide Hydrolase
mGluRs	Metabotropic Glutamate Receptors
mAChR	Muscarinic Acetylcholine Receptors
EPSPs	Excitatory Postsynaptic Potentials
DSE	Depolarization-induced Suppression of Excitation
IPSPs	Inhibitory Postsynaptic Potentials
iLTD	Long-term Depression of GABAergic Inputs
HFS	High Frequency Stimulation
LTD	Long-term Depression
PLC	Phosphoinositide-specific Phospholipase C
DAGL	Diacylglycerol Lipase
M1/M3 mAChR	Muscarinic Receptor Subtypes 1 and 3
PLCβ1	Phospholipase C subtype β1
GPCRs	G-Protein Coupled Receptors
GTPγS	Guanosine 5′-O-(3-thiotriphosphate)
MGL	Monoacylglycerol Lipase
DAG	Diacylglycerol
BLA	Basolateral Amygdala
TTX	Tetrodotoxin
mEPSPs	Miniature Excitatory Postsynaptic Potentials
LFS	Low Frequency Stimulation
DHPG	Dihydroxyphenylglycine
AC	Adenylyl Cyclase
PKA	Protein Kinase A
D1/D2	Dopamine Receptor Subtypes 1 and 2
AMPA	α-amino-5-hydroxy-3-methyl-4 isoxazole proprionic acid
VTA	Ventral Tegmental Area
NMDA	N-methyl-D-aspartic acid

1 Introduction

The endocannabinoids (eCBs) are a novel group of neuroactive lipids that play a recently revealed, important role in synaptic signaling (see [1] for review). The most well-studied eCBs are *N*-arachidonylethanolamine (anandamide, AEA) [2], 2-arachidonoylglycerol (2-AG) [3], and noladin ether [4]. These compounds

are agonists of cannabinoid receptors and are found in nervous tissue of many species. Myriad recent studies suggest widespread roles for this signaling system in synaptic physiology. In particular, a large body of work has accumulated that demonstrates the effects of eCBs on synaptic signaling in the hippocampus. The hippocampus is an attractive area in which to study both cannabinoid effects and neurotransmission because this region contains very high amounts of CB1 cannabinoid receptors (CB1R), has a well-defined synaptic circuitry, and expresses several forms of well-studied synaptic plasticity, including long-term potentiation (LTP) and depolarization-induced suppression of inhibition (DSI). In addition, the hippocampus is readily accessible for in vitro slice preparations and neuronal culture systems. In this chapter, we will discuss: (1) the localization and function of CB1Rs in the hippocampus; (2) the role of eCB signaling in short- and long-term forms of synaptic plasticity within the hippocampus; and (3) our current understanding of the determinants of eCB synthesis within hippocampal neurons. Lastly, we will briefly discuss other brain regions and neuronal circuits in which eCB signaling contributes to synaptic plasticity, including the cerebellum, amygdala, striatum, and ventral tegmental area.

2 Hippocampal CB1R Signaling

2.1 Localization and Function of CB1R in Hippocampus

The hippocampus expresses high levels of both CB1R mRNA and protein [5–13]. There is evidence for both pre- and postsynaptic localization of CB1Rs within the hippocampus, although most focus has been on the function of the presynaptic receptors. Within the hippocampus, a subset of cholecystokinin (CCK)-positive, GABAergic, inhibitory interneurons express high levels of CB1R [8, 10], while pyramidal neurons (which are glutamatergic) express low levels of CB1R [5, 6, 10, 13, 14]. The following section will detail the anatomical localization of CB1Rs and their function as modulators of afferent neurotransmitter release.

The first detailed study of the localization of the CB1R in hippocampus was published by Freund and co-workers [10]. They found CB1R-immunoreactive (CB1R-ir) neurons within all subfields of the hippocampus; in particular, strong somatic and dendritic staining was observed in interneurons. In addition, a dense meshwork of labeled axon terminals was found to surround pyramidal cell bodies [10]. The CB1R-ir interneurons coexpress the neuropeptide CCK, but not parvalbumin (PV), indicating the CB1R-ir interneuron population belongs to the CCK-containing, basket cell class that form perisomatic contacts with pyramidal neurons. Colocalization of CCK and CB1R is also seen at the ultrastructural level with CB1R-ir axon terminals also expressing CCK, but not PV [10]. Within the pyramidal layer, CB1R-ir was observed on the plasma

membrane of presynaptic axon terminals that form symmetric (inhibitory) contacts with postsynaptic elements [10]. Interestingly, CB1R-ir is predominantly extrasynaptic around axon butons at variable distances from the synaptic cleft. These data indicate that CB1Rs are ideally positioned to modulate presynaptic processes, including neurotransmitter release from GABAergic interneurons.

In accord with the anatomical localization of CB1Rs to GABAergic terminals, CB1R activation by the synthetic agonist Win 55212-2 concentration-dependent inhibits field-stimulation-induced GABA release (which is dependent upon action potentials) from hippocampal slices [10]. Pharmacological studies indicate that inhibition of GABA release by Win 55212-2 is dependent upon CB1R but not postsynaptic glutamatergic transmission. Win 55212-2 did not affect GABA uptake in this system [10]. Further studies by Hoffman and Lupica demonstrate that Win 55212-2 inhibits $GABA_A$ receptor-mediated, stimulation-evoked, inhibitory postsynaptic potentials (eIPSPs) in CA1 pyramidal neurons in a CB1R-dependent manner [15]. Win 55212-2 also inhibits spontaneously occurring, action potential-dependent, IPSPs (sIPSPs), but not action potential-independent, miniature IPSPs (mIPSPs) [15]. Win 55212-2 does not alter postsynaptic $GABA_A$ receptor responsiveness or GABA clearance [15]. These data are consistent with a mechanism by which presynaptic CB1R-activation results in suppression of GABA release, resulting in inhibition of GABAergic neurotransmission. This is revealed as a loss of stimulus-evoked, GABA-mediated IPSPs without an effect on spontaneous release of GABA.

2.2 *Endocannabinoid-Mediated Synaptic Plasticity in Hippocampus*

2.2.1 Depolarization-Induced Suppression of Inhibition (DSI)

DSI within the hippocampus was first described in the early 1990 s by Alger and co-workers. Using whole-cell patch-clamp recordings from hippocampal CA1 pyramidal neurons, these authors demonstrated that brief trains of action potentials rapidly and transiently suppressed $GABA_A$ receptor-mediated IPSPs onto these neurons [16–18]. They named this phenomenon “depolarization-induced suppression of inhibition” (DSI). Subsequent studies revealed that DSI was dependent upon calcium entry through L-type voltage-dependent calcium channels (VDCC) of the pyramidal neurons that could be initiated by multiple stimuli, including depolarizing voltage steps and trains of action potentials [16, 18]. DSI was not due to changes in the responsiveness of postsynaptic $GABA_A$ receptors, but did require intact G-protein signaling in the presynaptic neuron [16, 19]. Taken together, these data demonstrated that DSI is induced postsynaptically via depolarization, requires increased intracellular calcium in the postsynaptic neuron, and is expressed presynaptically as a reduction in quantal GABA release via a G-protein-dependent mechanism.

This sequence of events implies involvement of a retrograde signaling process that is initiated in the postsynaptic neuron and travels to presynaptic terminals to modulate afferent neurotransmitter release [20].

Three key observations regarding the mechanism of DSI and the biochemistry of eCB/CB1R signaling formed the basis for the hypothesis that DSI is mediated by eCB signaling. First, both DSI and eCB synthesis depend upon increases in intracellular calcium. Second, DSI is expressed presynaptically as a reduction in GABA release, and CB1R activation reduces quantal GABA release at interneuron-CA1 pyramidal neuron synapses. Third, DSI is dependent upon a $G_{\alpha i/o}$ signaling pathway in presynaptic terminals, and CB1Rs couple to the $G_{\alpha i/o}$ class of G proteins. Based on these similarities, several groups simultaneously tested the hypothesis that DSI was mediated by eCB signaling.

Wilson and Nichol were the first to report that eCB/CB1R signaling was involved in DSI at CA1 pyramidal neuron afferents [21]. These authors showed that DSI was suppressed in the presence of the CB1R antagonists SR141716 and AM251. In a subsequent manuscript, these authors demonstrated that DSI is absent in CB1R null mice, further solidifying a role for eCBs in the mediation of DSI [22]. DSI is both mimicked and occluded by the CB1R agonist, Win 55212-2, which provides evidence that Win 55212-2 and DSI inhibit GABA neurotransmission by a common mechanism [21]. The fact that Win 55212-2 and DSI increase the paired-pulse ratio (PPR) and decrease spontaneous, calcium-dependent, mIPSPs indicates a reduction in presynaptic vesicular release probability. DSI is not affected by botulinum toxin applied to the postsynaptic neuron, which indicates that the retrograde messenger does not utilize a synaptic release mechanism. This finding is consistent with what is currently understood about eCB synthesis and release, that is, eCBs are formed on demand from membrane precursors and do not utilize vesicular release for efflux [1]. Finally, these authors found that photo-uncaging of calcium within the postsynaptic neuron-mimicked DSI that was suppressed by CB1R antagonists [21], which suggests an increase in intracellular calcium is sufficient to mimic CB1R-dependent DSI. SR141716 alone does not affect eIPSPs, which suggests little tonic activation of CB1Rs by eCBs occurs under basal conditions. However, if eCB inactivation processes are blocked (via inhibition of fatty acid amide hydrolase (FAAH) activity or eCB accumulation into neurons) by AM404, depression of eIPSPs is observed, an effect blocked by SR141716. These findings suggest that eCB synthetic and catabolic processes are ongoing, but balanced, during basal conditions. However, when eCB catabolic processes are disrupted, DSI can be initiated (by accumulation of synaptically available eCBs) in the absence of postsynaptic depolarization.

The mechanism by which CB1R results in inhibition of GABA release is likely mediated by a direct effect of $G_{\beta\gamma}$ subunits to inhibit N-type VDCC [23, 24]. Alger and co-workers also studied the presynaptic factors that regulate DSI expression in hippocampus and concluded that DSI is mediated by reduced N-type VDCC activity downstream of CB1R activation, although a role for

increased potassium channel activation cannot be completely excluded [25]. Taken together, these data are consistent with the mechanisms by which CB1R activation inhibits neurotransmitter release.

The induction of DSI is affected by activation of G-protein-coupled receptors on the postsynaptic neuron. Alger and co-workers demonstrated that activation of metabotropic glutamate receptors (mGluRs)-enhanced DSI, while blockade of mGluRs reduced DSI [26]. This enhancement of DSI by mGluRs was blocked by the CB1R antagonist AM251, and absent in CB1R$^{-/-}$ mice, indicating that the enhancing effect of mGluR activation on DSI, like DSI itself, is dependent upon CB1R activation [26]. These data support a mechanism by which activation of mGluR1 on postsynaptic neurons facilitates eCB synthesis, likely 2-AG (see Sect. 2.3 below). Kano and co-workers came to similar conclusions based on finding that suppression of IPSPs by mGluR1 activation in hippocampal cell cultures and slices is dependent upon CB1R activation [27]. Importantly, this study demonstrated that all hippocampal neurons in culture expressing mGluR1-dependent synaptic suppression also showed CB1R-dependent inhibition of GABAergic transmission. Similarly, most neurons that did not exhibit mGluR1-dependent synaptic suppression also did not show CB1R-dependent inhibition of GABAergic transmission [27]. These data confirm a tight link between mGluR1 activation of eCB synthesis and/or release.

Activation of muscarinic cholinergic receptors (mAChR) also facilitates DSI induction in hippocampal CA1 pyramidal neurons. Application of the mAChR agonist carbachol enhanced DSI; an effect reversed by atropine [28]. Carbachol was unable to elicit or enhance DSI in slices from CB1R$^{-/-}$ mice or in slices treated with CB1R antagonists. These data suggest that mAChR activation also evokes eCB synthesis. Application of an acetylcholine esterase inhibitor facilitated DSI and reduced eIPSPs in a CB1R- and mAChR-dependent manner, suggesting that physiological concentrations of acetylcholine can enhance eCB synthesis and/or release and thereby facilitate DSI [28]. Investigations by Kano and co-workers revealed that both M1 and M3 mAChR receptors are involved in facilitation of eCB-mediated DSI [29]. Taken together, these data highlight the potential for cooperativity between depolarization and postsynaptic G-protein receptor activation in the initiation of eCB-mediated synaptic suppression. Possible mechanisms subserving the interactions between depolarization and G-protein-coupled receptor activation-induced eCB synthesis and release will be discussed below.

With regard to the functional, cellular, and anatomical specificity of hippocampal DSI, several interesting observations have been made. Nicoll and co-workers demonstrated that CB1R activation inhibits only a subset of GABAergic inputs onto CA1 pyramidal neurons [22]. These inputs are formed by interneurons that exhibit strong connections with pyramidal neurons, generating unitary IPSPs in postsynaptic neurons that are four times greater than cannabinoid insensitive synapses. The cannabinoid-sensitive interneurons display fast-spiking patterns of reactivity, form synapses onto the somata and

proximal dendrites of pyramidal neurons, and use exclusively N-type VDCC to trigger neurotransmitter release [22]. This last characteristic could explain the profound inhibition of neurotransmitter release induced by CB1R activation since N-type VDCC are twice as sensitive to inhibition by $G_{\beta\delta}$ subunits than the P/Q-type (which are expressed only by cannabinoid-insensitive synapses) [22]. It is not surprising that only a subset of GABAergic inputs to pyramidal neurons are CB1R-sensitive given the restricted anatomical localization of CB1R to the subset of CCK-expressing GABAergic interneurons within the hippocampus [8, 10].

It has been proposed that these fast-spiking, CB1R-sensitive interneurons control γ band oscillations [30], which are responsible for binding simultaneous perceptions and are observed during the coordinated activities of neuronal networks across multiple brain regions [31]. In support of this, exogenous activation of CB1Rs reduces the power of γ band oscillations induced by kainic acid in hippocampal slices [9]. Thus, hippocampal eCB signaling could be involved in fundamental aspects of attention, perception, and consciousness.

With regard to anatomical specificity, Isokawa and Alger found that granule neurons of the hippocampal dentate gyrus exhibit DSI. This DSI has characteristics similar to CA1 pyramidal neurons, including requirements for increased intracellular calcium and activation of CB1R [32]. DSI in this region was also observed after the induction of febrile seizures in rats [33]. In contrast, Lupica and co-workers reported that synapses between GABAergic interneurons of the CA1 region do not express DSI [34]. Furthermore, coapplication of mAChR agonists (which facilitate DSI induction in CA1 pyramidal neurons [28]) does not induce DSI at GABAergic–CA1 interneuron synapses. Since a subset of GABAergic synapses onto CA1 interneurons do express functional presynaptic CB1R, these data suggest that hippocampal interneurons, in contrast to pyramidal and granule neurons, do not synthesize eCBs in response to depolarization [34].

While the elucidation of eCBs as mediators of hippocampal DSI imparts the eCB/CB1R signaling system with a prominent role in synaptic physiology, these data beg the question of the behavioral significance of hippocampal DSI. The answer to this question would correlate in vitro synaptic physiology with neural substrates of behavior. Hampson, Deadwyler, and co-workers utilized a simple, direct approach to explore the functional significance of hippocampal DSI [35]. They recorded neuronal activity from hippocampal pyramidal neurons while animals exhibited two different hippocampal-dependent behaviors. In the first behavioral paradigm, the activity of hippocampal neurons that code for a specific place fields were measured in awake, behaving rats. In the second, firing rate of hippocampal neurons was determined prior to lever pressing in a hippocampal-dependent memory task (delayed nonmatch to sample). The firing rates displayed under these physiological conditions (0.5–10 Hz for place fields, and 0.5–17.8 Hz for the memory task) were subsequently tested for their ability to elicit DSI in hippocampal CA1 neurons in vitro. Surprisingly, these neuronal activity patterns were unable to induce DSI in vitro [35]. In

investigations of the factors that would overcome the frequency constraints of DSI in hippocampal neurons, the authors found that temporal summation of multiple, subthreshold (for DSI), asynchronous inputs to pyramidal neurons could trigger activation of DSI [36]. Neuronal simulation studies revealed that, at a firing frequency of 10 Hz, three separate but temporally overlapping inputs could allow the postsynaptic neurons to reach threshold for activation of DSI [36]. Increased intracellular calcium is the trigger for DSI, which occurs as a common endpoint following activation of multiple excitatory neurotransmitter systems [36]. These authors conclude that DSI occurs when hippocampal pyramidal neurons receive multiple, coincident excitatory inputs regardless of the specific neurotransmitter system involved.

Alger and co-workers utilized an electrophysiological approach to examine the functional significance of DSI in hippocampal CA1 pyramidal neurons. These authors found that weak trains of stimuli delivered to pyramidal neurons did not produce LTP of excitatory postsynaptic potential (EPSPs); however, when a depolarizing pulse preceded the same stimulation protocol, LTP was induced [37]. This finding suggested that the depolarizing prepulse induced DSI, and the suppression of inhibition allowed previously ineffective excitatory inputs to induce LTP. DSI-induced facilitation of LTP was abolished by the CB1R antagonist AM251, leading these authors to hypothesize that eCB signaling, through DSI, could facilitate LTP in neurons receiving multiple or repetitive inputs and thereby contribute to memory formation [37].

DSI represents the first-identified physiological role for eCBs in synaptic signaling. We have discussed the discovery of eCBs as mediators of DSI, the functional, cellular, and anatomical selectivity of the DSI process within the hippocampus, and the potential functional significance of this type of eCB-mediated synaptic signaling. Although much work has been done at the cellular level, future studies will likely be aimed at elucidating the contribution of DSI to hippocampal-dependent processes such as memory formation and epileptogenesis.

2.2.2 Depolarization-induced Suppression of Excitation (DSE)

Like DSI, depolarization-induced suppression of excitation (DSE) has been investigated in the hippocampus using both slice preparations and cell culture systems and found to involve eCB/CB1R signaling. Specifically, depolarization of CA1 pyramidal neurons results in transient suppression of EPSP amplitude that is dependent upon CB1R activation [38]. Like DSI, DSE also increases the PPR, indicating a presynaptic site of action, is blocked by the CB1R antagonists AM281 and SR141716, and is absent in CB1R$^{-/-}$ mice [38].

However, in contrast to DSI in CA1 pyramidal neurons, DSE requires longer depolarization times (10 s for DSE vs. 5 s for DSI) and the magnitude of DSE (with an appropriately longer depolarizing step) is approximately half that of DSI under otherwise identical experimental conditions [38]. Since eCBs mediate both phenomena and their synthesis/release is being evoked by

essentially the same stimulus, these data suggest that presynaptic CB1R signaling is less efficacious when the receptor is on glutamatergic terminals than on GABAergic terminals. In accord with this hypothesis, CB1R that regulate glutamate release are 10–30-fold less sensitive to agonist activation that those that regulate GABA release. In one study, the IC50 value for Win 55212-2 to inhibit EPSPs was 60 nM while the value for IPSP inhibition was 2 nM [38]. Similarly, in another study, the Win 55212-2 IC50 value for EPSP inhibition was 2.1 μM versus 0.24 μM for IPSP inhibition [39]. A second difference between DSI and DSE lies in the number of neurons in a slice that display the plasticity. Data from Hajos and Freund indicate that CB1R activation is more efficacious in reducing EPSPs than IPSPs ($E_{\max}$ 100% inhibition for EPSPs vs. $E_{\max}$ 50% inhibition of IPSPs) [39]. These data are in agreement with findings that glutamatergic terminals are more homogeneous and uniformly sensitive to CB1R inhibition than GABAergic terminals, of which approximately 50% are sensitive to CB1R inhibition [38].

DSE has also been studied in autopic hippocampal cultures, neurons that form synaptic connections upon themselves [40]. Depolarization of cultured neurons for 1–10 s reduces EPSP amplitude for more than 1 min. This effect is blocked by the CB1R antagonist SR141716 and is absent in cultures obtained from CB1R$^{-/-}$ mice. DSE also increases the PPR indicating a presynaptic site of expression and is dependent upon intracellular calcium release [40]. Taken together, these studies indicate that eCB signaling mediates DSE in hippocampal pyramidal neurons with many similarities to DSI [38, 40]. The in vivo functional significance of this type of eCB-dependent synaptic suppression remains to be determined.

2.2.3 eCB-Mediated Long-Term Depression of GABAergic Inputs (iLTD)

While eCB-mediated DSI and DSE are transient forms of synaptic plasticity, eCBs have also been implicated in longer, more persistent forms of synaptic plasticity. In particular, Chevaleyre and Castillo demonstrated that stimulation protocols which induce LTP of excitatory synapses onto CA1 pyramidal neurons also produce long-term depression of GABAergic inputs to the same neurons; they called this phenomenon iLTD [41]. High-frequency stimulation (HFS) of inputs to CA1 pyramidal neurons depresses GABAergic transmission onto these neurons for almost 1 h, an effect that is presynaptic in nature, and requires activation of mGluR1. Importantly, iLTD is blocked by CB1R antagonists applied during induction, indicating mediation by eCBs [41]. However, eCBs are not required for iLTD maintenance since application of CB1R antagonists 20 min after induction does not reverse iLTD. Furthermore, all synapses that express iLTD also express DSI, indicating that there is a relationship between DSI and iLTD and that this relationship involves mediation by eCB signaling [41]. These data suggest that HFS of excitatory inputs onto CA1 pyramidal neurons releases glutamate, which activates postsynaptic mGluR1 receptors, mGluR activation results in eCB synthesis and release, eCBs activate

presynaptic CB1R on GABAergic terminals resulting in short-term inhibition of GABA release, and, through an unknown mechanism initiates long-term depression of GABAergic inputs [41]. However, a problem with this hypothesis arises from the demonstration that CB1R antagonists also block mGluR1-induced iLTD, which should initiate the same signaling cascade, but it is the maintenance and not the initiation of iLTD induced by mGluR1 activation that are dependent upon CB1R. In other words, when HFS is used to induce iLTD, the initiation of the process is CB1R requiring, while initiation by mGluR1 activation requires CB1R for the long-term depression component. Interestingly, although persistent mGluR1 activation can induce eCB-mediated iLTD, prolonged activation of CB1R by eCBs (initiated by repeated depolarizations) does not induce iLTD, suggesting that mGluR1 activation also initiates an eCB-independent signaling pathway that is required for iLTD. In other words, eCB signaling is necessary but not sufficient for mGluR1-induced iLTD, suggesting another pathway contributes to this form of plasticity [42]. Clearly, the mechanism involved is more complex. The functional relevance of eCB-mediated iLTD has been explored. Chevaleyre and colleagues demonstrated that a low-frequency but highly localized stimulation protocol which induced iLTD can facilitate induction of LTP at synapses up to 10 μm away [43]. Thus, iLTD acts to *prime* nearby synapses to induction of LTP. This priming is abolished by CB1R antagonist application. Taken together, these data indicate that highly localized, excitatory inputs to CA1 pyramidal cell dendrites can initiate eCB release and induce iLTD in nearby synapses; this iLTD results in the facilitation of LTP induction in these and nearby synapses. Thus, one function of eCB-mediated iLTD could be to shape the anatomical delineation of subsequent LTP induction. These data suggest that global depolarization and somatic eCB production, which are used to produce DSI/E experimentally, do not mirror what happens in vivo where highly localized, focal areas of eCB signaling are more likely to occur. These findings likely have important implications for understanding the synaptic determinants of memory formation.

2.2.4 eCB-Mediated LTD at Excitatory Synapses

Like iLTD, long-term depression (LTD) of excitatory synaptic transmission also involves eCB signaling. Rouach and Nicoll examined the role of eCB signaling in mGluR1-mediated LTD at Schaffer collateral–CA1 pyramidal neuron synapses [44]. Activation of mGluR1 receptors produces both immediate and persistent depression of EPSPs. The immediate, but not the persistent, EPSP depression is blocked by the CB1R antagonist SR141716 [44]. Interestingly, neither component is blocked by the CB1R antagonist AM251 and the response to mGluR1 activation is unaltered in $CB1R^{-/-}$ mice, suggesting a non-CB1R but SR141716-sensitive receptor mediates the acute component of mGluR1-induced LTD. The identity of this receptor is unknown.

2.3 Determinants of eCB Synthesis and Release

We have reviewed functional data that are consistent with eCB production by postsynaptic neurons in response to stimulation protocols that induce the various forms of synaptic plasticity. These data also shed light on the biochemical determinants of hippocampal eCB synthesis and release, which we will now discuss. With few exceptions, the mechanisms involved in synthesis and release cannot be discriminated using functional endpoints, so the term synthesis/release will be used to refer to the combined process. One interesting and surprising outcome of a comparison of available data is multiple stimuli that can induce CB1R-dependent changes in synaptic plasticity do not all induce eCB synthesis/release by the same mechanism. This leads to the conclusions that multiple eCBs are utilized in these processes, and synthesis and release are separately regulated steps. Possibly, synthesis and release can occur via multiple pathways. We will also discuss data regarding the temporal dynamics of eCB signaling in hippocampus.

As was discussed above, HFS of Schaffer collaterals induces LTD and iLTD, components of which require eCB synthesis/release. Initial investigations into the determinants of eCB synthesis in hippocampus were conducted by Piomelli and co-workers [45]. They found that HFS of Schaffer collaterals, which are excitatory inputs to CA1 pyramidal neurons, increases the content of 2-AG, but not AEA, in hippocampal slice preparations. The increase in 2-AG content requires action potential-dependent neurotransmitter release and extracellular calcium. Pharmacological data indicate that 2-AG synthesis requires activation of both phosphoinositide-specific phospholipase C (PLC), and diacylglycerol lipase (DAGL), which is consistent with a pathway in which 2-AG would be synthesized [46]. Chevaleyre and Castillo explored the postsynaptic determinants of eCB synthesis in response to HFS of Schaffer collaterals indirectly, using the expression of iLTD to infer eCB synthesis/release [41]. They found that HFS-induced iLTD (and thus, presumably, eCB synthesis/release) did *not* require an elevation in intracellular calcium, but was dependent upon PLC and DAGL activity. Taken together, these data suggest that 2-AG is the primary eCB produced in response to HFS of excitatory afferents to CA1 pyramidal neurons and, in this situation, does not require elevations in intracellular calcium for its synthesis/release.

The stimulus for DSI and DSE is postsynaptic depolarization, leading to the hypothesis that milder conditions compared to HFS also induce eCB synthesis/release. Several investigators have demonstrated a requirement for increased intracellular calcium for the expression of DSI, which suggests that increased intracellular calcium is required for eCB synthesis/release [16, 21, 32, 47–49]. In fact, increased postsynaptic calcium is both necessary and sufficient since DSI can be mimicked by liberating intracellular calcium using a photo-uncaging technique [21, 50] and replacement of extracellular calcium with strontium maintains DSI [49]. The calcium required for eCB synthesis/release in response

to depolarization can come from either external influx via L-type VDCC [16, 51] or release of ryanodine-sensitive, intracellular calcium stores [32, 52]. It is likely that the full-expression DSI requires depolarization-induced activation of VDCC, which initiates calcium-induced calcium release from ryanodine-sensitive stores. Interestingly, DSE in hippocampus is also calcium-dependent but is driven more by release of intracellular calcium than VDCC opening [40].

When DSI is used as a bioassay for eCB synthesis/release there is no additional requirement for PLC or DAGL activation [41, 53]. However, when DSE is used as a bioassay for eCB synthesis/release in a cultured autaptic system [40], intact DAGL activity is required. Since DAGL is required for 2-AG but not AEA synthesis, it is likely that 2-AG is the eCB-mediating hippocampal DSE. In summary, eCB synthesis/release in response to depolarization is dependent upon both extra- and intracellular calcium, but is not dependent upon PLC or DAGL activity when DSI is used as the bioassay. In contrast, when DSE is used as a bioassay, eCB synthesis/release appears to be regulated primarily by intracellular calcium release and DAGL activity. Thus, the identity of the eCB-mediating DSI remains unknown (as calcium is requirement for both AEA and 2-AG synthesis), but the eCB-mediating DSE is likely 2-AG.

As noted in the previous section, activation of mGluR1 and M1/M3 mAChR can induce eCB-mediated inhibition of GABAergic and facilitate DSI. Activation of mGluR1 without depolarization induces eCB-mediated suppression of IPSPs, an effect that is absent in PLC$\beta 1^{-/-}$ mice but, curiously, not blocked by pharmacological inhibitors of PLC or DAGL, and is independent of increases in intracellular calcium [27, 42]. mGluR1-mediated facilitation of DSI (and, therefore, eCB synthesis/release) is not due to enhancement of depolarization-induced intracellular calcium release [27]. Persistent activation of mGluR1 is also required for eCB-mediated iLTD, and this effect was dependent upon non-postsynaptic (i.e., extracellular or presynaptic) PLC and DAGL activities [41, 42]. Taken together, these data suggest that the primary effect of mGluR1 activation is to increase 2-AG synthesis/release via a non-calcium-dependent mechanism which can mimic DSI without the need for depolarization; or can synergize with depolarization-induced DSI, which acts through a calcium-dependent process. Kano and co-workers suggest that the synergism is mediated by PLCβ1 acting as a coincidence detector, since it is activated synergistically by the combination of GPCR-mediated signaling and increased intracellular calcium, resulting in enhanced eCB synthesis/release [53].

Like mGluR activation, M1/M3 mAChR also induce eCB-mediated synaptic suppression by a mechanism that is not dependent upon increases in intracellular calcium [28, 29]. In fact, generalized activation of G-protein signaling via postsynaptic application of GTPγS produces eCB-dependent synaptic suppression that occludes both mGluR and mAChR-induced synaptic suppression [28]. There are similarities and differences in the biochemical events involved in eCB synthesis/release between mAChR and mGluR1 activation. In contrast to mGluR1, eCB synthesis/release is dependent upon postsynaptic DAGL activity, but like the mGluR1 system, is not sensitive to inhibition of PLC activity

[42]. Like mGluR1, mAChR facilitation of DSI is not dependent upon PLC or DAGL activity [42], but it was found to be absent in PLCβ1$^{-/-}$ mice [53].

With regard to the contribution of the eCB transport process to eCB-dependent synaptic signaling, Straiker and Mackie explored the role of the eCB transporter in DSE using autaptic hippocampal culture system [40]. They found that bath application of the eCB-transport inhibitor UCM-707 inhibited DSE, but did not affect the time-course of exogenously applied 2-AG to inhibit EPSPs. When UCM-707 was applied selectively to the inside of postsynaptic neurons, a slight reduction of DSE was observed. These data are consistent with the hypothesis that a membrane eCB transporter regulates efflux of eCBs from hippocampal pyramidal neurons [54], with minimal effect on inactivation of exogenously added 2-AG.

The temporal dynamics of eCB signaling in hippocampus has also been investigated, using a novel photolytic AEA release system [55]. The aim of the study was to estimate the time required for eCB synthesis and release in response to action potential trains that induce DSI. The following equation was used: $T_{\mathrm{DSI}} = T_{\mathrm{Ca}} + T_{\mathrm{eCB}} + T_{\mathrm{CB1}}$, where T_{DSI} represents the time to onset of DSI after afferent excitatory stimulation, T_{Ca} represents time required for intracellular calcium concentrations to reach threshold for eCB synthesis, T_{eCB} represents the time required for eCB synthesis and release, and $\mathrm{T_{CB1}}$ represents the time between CB1R activation and inhibition of GABA release. T_{DSI} was empirically determined to be about 350 ms. T_{Ca} was estimated at 60 ms based on measurements of intracellular calcium changes that initiate eCB synthesis/release as assayed by the induction of DSI in response to direct postsynaptic depolarization. T_{CB1} was estimated by the use of photolytic AEA release system. The time from photo-activation to detection of CB1R-dependent inhibition of IPSPs was 162 ms (T_{CB1}). Using the equation above, T_{eCB}, which is the time remaining for eCB synthesis, release, and diffusion from postsynaptic to presynaptic neuron, was estimated to be 187 ms [55]. Similar measurements were made using mGluR1-driven synaptic suppression and a value of $T_{\mathrm{eCB(mGluR)}} = 74$ ms was determined [55]. These findings place significant temporal constraints on eCB signaling, which suggests a rapid synthesis and/or release process must be in play despite the multiple enzymatic steps thought to mediate eCB synthesis. These calculations call into question the assumption that eCB release is completely synthesis dependent; perhaps a pool of pre-synthesized eCB is immediately mobilized upon mGluR activation or depolarization, which is followed or replenished by *de novo* synthesis.

An important and open question is the chemical identity or identities of the eCB involved in retrograde inhibition. Two studies have explored this question indirectly using selective inhibitors of AEA and 2-AG catabolism [56, 57]. The rationale for these studies is that if a catabolic enzyme inhibitor prolongs the duration of retrograde inhibition, a substrate of that catabolic enzyme subserves the retrograde inhibition. Both studies used hippocampal slices and both demonstrated that a selective inhibitor of FAAH, URB597, did not affect DSI [56, 57]. Although FAAH hydrolyzes both AEA and 2-AG in vitro, its

inhibition in vivo only increases AEA content [58]. The authors argued that the lack of effect of FAAH inhibition suggests that AEA is not involved in the hippocampal DSI. However, an alternative explanation would be that FAAH is not involved in the inactivation of AEA under these conditions. On the other hand, inhibition of monoacylglycerol lipase (MGL), which hydrolyzes 2-AG and not AEA [59], did prolong hippocampal DSI [57], suggesting that 2-AG is the relevant eCB in this context.

In summary, depolarization-induced eCB synthesis/release is mediated via increases in intracellular calcium, but does not consistently require PLC or DAGL activity. In contrast, pharmacological studies indicate that GPCR-driven eCB synthesis/release does not require increased intracellular calcium or PLC activity, although mAChR-mediated eCB synthesis requires DAGL activity. GPCR facilitation of eCB-mediated DSI is not mediated via augmentation of depolarization-induced increased intracellular calcium, which is consistent with an effect on PLC activation. That DSI does not require the activity of enzymes required for 2-AG synthesis suggests another eCB may be involved, or that depolarization results in release of a preformed pool of 2-AG or DAG, whereas GPCR-induced eCB synthesis may rely on a *de novo* synthetic pathway – at least in the case of mAChR-induced eCB synthesis [42]. However, both mGluR1- and mAChR-mediated synaptic suppression are absent in PLCβ1$^{-/-}$ mice. These data are consistent with the idea that at least a pool of eCBs are not synthesized on demand but pre-made and stored in a releasable form. Transient, pharmacological inhibition of the synthetic pathway would cause gradual loss of eCB-mediated functional effects while permanent deletion of the synthetic enzyme would abrogate signaling completely. This hypothesis is readily testable by measuring 2-AG content in the brains of PLCβ1$^{-/-}$ mice. In addition, an eCB transport process appears to contribute to synaptic eCB-mediated signaling in hippocampus. Lastly, eCB signaling in hippocampus is a very rapid event, suggesting fast synthesis and/or mobilization of eCBs is required for CB1R-mediated synaptic suppression.

3 eCB-Mediated Synaptic Plasticity in Other Brain Regions

3.1 Cerebellum

The density of CB1R is very high in the cerebellum. In particular, CBIR are expressed by all neurons that make synapses with Purkinje cells, that is, granule neurons, basket cells, and climbing fibers [60]. One of the first demonstrations of eCB-mediated DSI was at the synapse between basket cells, and cerebellar Purkinje neurons [61, 62]. A parallel modulation of excitatory inputs (DSE) has also been demonstrated at Purkinje cell synapses such that Purkinje cell depolarization induces short-lived inhibition of glutamate release from both parallel and climbing fiber inputs [63].

Activation of mGluR1 either by repetitive stimulation of parallel fibers [64, 65] (which results in glutamate release and postsynaptic mGluR1 activation) or by direct application of mGluR1 agonists [64–66] results in eCB-mediated DSE [64, 65] and DSI [66].

There is unequivocal, although indirect, evidence that eCBs are *the* retrograde messengers of cerebellar DSI/E [1, 63, 66–71]. DSI/E triggered by mGluR1 activation of Purkinje cells is also mediated by eCBs; it is completely inhibited by CB1R antagonists and occluded by CB1R agonists [64–66].

Both depolarization and GPCR-activation are physiological relevant triggers of eCB-mediated retrograde inhibition although their relative importance varies among brain regions and, possibly, synapse types. A study by Brown and colleagues [65] found that presynaptic depolarization of parallel fibers, which results in the release of glutamate, simultaneously induces both depolarization-triggered and mGluR1-triggered, eCB-mediated retrograde inhibition. This study demonstrated that retrograde inhibition occurs following physiologically relevant increases in presynaptic input and that both GPCR- and depolarization-induced retrograde inhibition require CB1R activation.

3.2 Amygdala

The amygdala contains high levels of CB1R and, within the basolateral amygdala (BLA), a distribution similar to the hippocampus is observed. High levels of CB1R expression have been demonstrated within CCK-positive GABAergic interneurons in this region, giving the BLA a cortical-like pattern [6, 72, 73]. Lower levels of CB1R expression have been observed within BLA pyramidal neurons [6, 7, 72]. The central amygdala has a lower density of CB1R than the BLA, similar to the density of other striatal areas [12, 74, 75]. At the synaptic level, CB1R activation has been demonstrated to modulate both GABAergic and glutamatergic neurotransmission in the BLA, and roles for eCB signaling in DSI, iLTD, and amphetamine-induced LTD in BLA have been recently demonstrated.

Cannabinoids have been shown to decrease evoked and spontaneous GABAergic synaptic transmission onto BLA principal neurons. Katona and colleagues demonstrated, using intracellular recordings from rat BLA principal neurons, that the amplitude of eIPSP are suppressed by application of the CB1R agonists Win 55212-2 and CP 55940 to 61% and 59% of control, respectively; both agonist effects are reversed by the CB1R antagonist SR141716 [73]. Win 55212-2 is unable to suppress GABAergic transmission within the BLA of CB1R$^{-/-}$ mice. CB1R activation also decreases the amplitude of eIPSP at synapses onto mouse lateral amygdala principal neurons. This effect is accompanied by an increase in the PPR, reversed by SR141716, and absent in CB1R$^{-/-}$ mice [76]. Therefore, activation of CB1R inhibits GABA release onto principal neurons in the BLA.

Recently, Zuh and Lovinger have utilized an isolated neuron/synaptic bouton preparation to examine the effects of CB1R activity on the regulation of GABAergic transmission onto BLA principal neurons [77]. Application of Win 55212-2 produces a rapidly reversible reduction in the frequency and amplitude of action potential-dependent sIPSPs, which is followed by a rebound increase in sIPSP frequency. Blockade of CB1R with either AM251 or SR141716 both increase sIPSP frequency. This effect of AM251 is not dependent upon action potentials since tetrodotoxin (TTX) did not eliminate the increase in IPSP frequency but is dependent upon calcium [77]. These data indicate that eCB signaling is operative in this preparation and serves to tonically inhibit GABAergic transmission onto BLA principal neurons.

The fact that CB1R activity inhibits GABA release suggests that eCBs mediate DSI at the BLA principal neurons. In the acute brain slice preparation, postsynaptic depolarization (–60 to 0 mV) of BLA principal neurons for 4 s produces DSI, seen as a 44% decrease in sIPSP frequency and a 34% decrease in sIPSP amplitude [77]. This depolarization protocol also suppresses eIPSPs to 37% of control. DSI is eliminated by application of the CB1R antagonist AM251. Interestingly, AM251 was also found to increase sIPSP frequency by 13% and 22% in two of five neurons tested; AM251 had no effect on the remaining neurons. DSI was also explored using the postsynaptic neuron/synaptic bouton using moderately elevated external potassium concentrations (9 mM) to evoke depolarization to 0 mV [77]. Compared to the 20 s before depolarization, there was a 50% reduction in the number of sIPSP during the 20 s after depolarization; sIPSP frequency did not recover to control levels for greater than 15 min. DSI in this preparation is abolished by application of the CB1R antagonist AM251 and by chelation of postsynaptic calcium; however, a slowly developing inhibition of sIPSP persists under these conditions. An mGluR5 antagonist does not eliminate DSI, but it does significantly shorten its duration suggesting that a long-lasting component of DSI requires mGluR5 receptor activation. Therefore, in accord with the process at pyramidal neurons in hippocampus, DSI at principal neurons in the BLA is CB1R dependent and eCB synthesis is calcium requiring.

In contrast to the BLA, no effect of CB1R activation on GABAergic transmission is observed within the central amygdala [73].

The effects of CB1R activation on glutamatergic transmission has also been studied in the amygdala [76]. Win 55212-2 reduces eEPSP amplitudes and increases the PPR at glutamatergic synapses onto principal neurons in the BLA. These effects of Win 55212-2 are absent in CB1R$^{-/-}$ mice. Win 55212-2 also reduced the frequency of spontaneous EPSPs and action potential-independent miniature EPSPs (in the presence of TTX) without affecting the amplitude of either response, further support for a direct effect on glutamate release. Interestingly, pharmacological studies indicate that the mechanism for CB1R inhibition of evoked EPSP (eEPSP) amplitude in the BLA involves increased potassium-channel activation rather than inhibition of calcium-channel opening, a mechanism that differs from that seen in the hippocampus. These data

indicate that CB1R activation decreased external capsule evoked excitatory glutamatergic transmission onto BLA principal neurons via a presynaptic mechanism involving potassium but not calcium channels or action potential-driven glutamate release [76].

In addition to the short-term modulation of GABAergic and glutamatergic transmission by CB1R activity, eCB signaling has been demonstrated to mediate other forms of synaptic plasticity onto principal neurons of the BLA. Two studies have examined the role of eCB signaling in iLTD within the mouse amygdala using intracellular recordings from BLA principal neurons [78, 79]. Marsicano, Lutz, and colleagues have demonstrated that iLTD is induced by low-frequency (1 Hz) stimulation (LFS) of the external capsule (100 pulses) and produces a decrease in isolated $GABA_A$ receptor-mediated IPSP amplitude to 66% of control, an effect which lasts for more than 20 min and is accompanied by an increase in the PPR [79]. In addition, LFS reduces sIPSP frequency but not amplitude, further suggesting a presynaptic mechanism of action [78]. LFS-induced iLTD is mediated by eCB signaling since it is absent in $CB1R^{-/-}$ mice, reversed by the CB1R antagonist SR141716, and occluded by Win 55212-2, which alone decreases eIPSP amplitude to 56% of control [78]. iLTD does not require postsynaptic elevations in intracellular calcium but is dependent upon postsynaptic G-protein activation [78]. In particular, there is good evidence that metabotropic glutamate receptors are the triggers for eCB synthesis/release. An mGluR agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) suppresses eIPSP amplitude in wild type but not $CB1R^{-/-}$ mice. DHPG occludes iLTD and does not affect eIPSP amplitude after iLTD has been induced. Brief application of DHPG (to mimic mGluR activation during iLTD induction) has similar effects. Pharmacological evidence indicates a specific role for mGluR1, rather than mGluR5. Interestingly, however, pharmacological evidence indicates that the adenylyl cyclase (AC)-protein kinase A (PKA) signaling pathway and not PLC leads to eCB synthesis/release in this preparation. Blockade of AC or PKA with SQ22,536 or Rp-cAMP, respectively, abolish iLTD while inhibition of PLC and DAGL have no effect [78]. These data indicate that iLTD in the BLA is not mediated by 2-AG, bringing up the possibility that AEA is the primary eCB at these synapses. In support of this hypothesis, $FAAH^{-/-}$ mice, in which brain *N*-acylethanolamine contents, including AEA are elevated [58], exhibit pronounced iLTD compared to wild-type mice. Taken together, these data suggest that mGluR1, acting via an AC-PKA pathway, induces AEA-dependent iLTD in the BLA [78]. This is a very interesting finding as it adds to the pool of G-protein-coupled receptors that are apparently capable of inducing eCB-mediated signaling.

A role for eCB signaling in amphetamine-induced LTD of external capsule-evoked EPSPs within BLA principal neurons has also been demonstrated [80]. Application of amphetamine causes a rapid, dose-dependent reduction in eEPSP slope with both an early acute and late component (i.e., LTD). Blockade of D2 dopamine receptors partially reduces the acute depression, while D1 and D2 dopamine receptor agonists mimic the acute effects of amphetamine when

applied alone. Amphetamine-induced LTD is unaffected by D2 receptor ligands and is intact in slices from dopamine-depleted rats. These data suggest the acute depression in eEPSP slope observed after amphetamine application is partially dependent upon dopamine receptor activation while the LTD is not.

The CB1R antagonist AM251 blocks both the acute depression and LTD induced by amphetamine, while having no effect on eEPSP slope when administered alone. The CB1R agonist, Win 55212-2, mimics both the initial depression and LTD produced by amphetamine, an effect that is abolished by AM251. Win 55212-2 occludes the effects of amphetamine while amphetamine occludes the effects of Win 55212-2 to produce LTD. Similarly, an inhibitor of eCB inactivation, AM404 [81], mimics amphetamine- and Win 55212-2-induced LTD at low concentrations and occludes amphetamine LTD at high concentrations. These findings suggest that amphetamine, Win 55212-2, and eCBs all share a common mechanism of action to induce LTD. Both amphetamine and Win 55212-2 increase PPR and reduce mEPSP frequency (recorded in the presence of TTX, and mediated by AMPA receptors) but not amplitude, consistent with inhibition of glutamate release as the mechanism of action of both agents. However, Win 55212-2 but not amphetamine inhibits potassium-stimulated glutamate release from purified amygdala synaptosomes, suggesting amphetamine affects presynaptic glutamate release indirectly, perhaps via eCB mobilization. Taken together, these data strongly suggest amphetamine-induced LTD is mediated by eCB signaling which regulates glutamate release onto BLA principal neurons [80].

The mechanism by which amphetamine increases eCB synthesis/release is not completely clear. Amphetamine-induced acute depression of eEPSPs is partially dependent upon D2 receptor activation, which is known to increase brain AEA contents [82, 83]. However, amphetamine-induced LTD is completely independent of dopamine receptor activation, suggesting another pathway to eCB synthesis/release must occur. Amphetamine-induced LTD is calcium dependent; however, the precise mechanism by which amphetamine activates eCB-mediated LTD remains to be explored.

In summary, there is compelling evidence that presynaptic CB1R activation reduces both GABAergic and glutamatergic transmission at afferents to BLA principal neurons by mechanisms that are very similar to those seen in the hippocampus. Interestingly, however, biochemical evidence suggests that the mechanisms involved in the synthesis/release of eCBs in the BLA differ. In particular, eCB-mediated iLTD appears to depend on AEA, not 2-AG, is not regulated by postsynaptic calcium and requires a functional AC-PKA pathway. In contrast, eCB-mediated DSI is dependent upon elevations in postsynaptic calcium and its duration depends on mGluR5 activation; the eCB ligand involved in DSI has not yet been explored. Lastly, amphetamine-induced LTD involves eCB signaling, however, the mechanisms by which amphetamine activates eCB signaling remain to be determined.

What are the functional implications of cannabinoid regulation of afferent neurotransmission within the BLA? One hypothesis [73, 78] is that when BLA

pyramidal neurons are activated by incoming excitatory inputs, eCBs are released and inhibit local GABAergic transmission. This potentiates the activity in BLA projection neurons and increases the activation of target nuclei such as the central amygdala, nucleus accumbens, striatum, and prefrontal cortex. Enhanced BLA output would thus facilitate instrumental, autonomic, and endocrine responses to salient stimuli. This *dysinhibition* model has received some support from in vivo studies demonstrating that low doses of CB1R agonists facilitate activation of the central amygdala by restraint stress [75].

3.3 Ventral Tegmental Area (VTA) Dopamine Neurons

Mesolimbic dopamine systems, which include dopaminergic neurons of the VTA and their projections to limbic forebrain targets, including the nucleus accumbens, amygdala, and prefrontal cortex, have a primary role in motivation, reward learning, and working memory. Dysregulation mesolimbic dopaminergic circuits occurs in a wide variety of neuropsychiatric disorders including schizophrenia [84], addiction [85], and depression [86]. Since cannabis intoxication in humans affects motivation and mood, is rewarding, and can precipitate psychotic symptoms in susceptible individuals, it has been hypothesized that cannabinoid interactions with VTA dopamine neurons occur (see [87] for review). These observations beg the question: "Does eCB-mediated synaptic plasticity occur in the VTA?" Afferent inputs to the VTA dopaminergic neurons from the prefrontal cortex, nucleus accumbens, and pontine tegmental nuclei have been demonstrated to express CB1R [5, 6]. However, there is no evidence that CB1R is expressed by VTA dopamine neurons [88], nor has CB1R immunoreactivity been detected within afferent fibers innervating, or local GABAergic neurons within the VTA. In spite of the apparent lack of CB1R expression in this brain region, several studies have now demonstrated synaptic effects of CB1R activation within the VTA.

In studies of the synapse between GABAergic interneurons and presumptive VTA dopamine neurons, Szabo and colleagues found that Win 55212-2 inhibits eIPSP amplitude via a presynaptic, CB1R-dependent mechanism that is independent of postsynaptic alterations in $GABA_A$ receptor responsivity [89]. Riegel and Lupica have also demonstrated that Win 55212-2 inhibits stimulation-evoked $GABA_B$ receptor mediated IPSPs in VTA dopamine neurons [90]. However, only 50% of neurons tested were affected by Win 55212-2, suggesting only a subset of GABAergic terminals that form synaptic contacts with VTA dopamine neurons are CB1R-sensitive. This effect was blocked by AM251. CB1R activation also inhibits excitatory inputs onto VTA dopamine neurons. The CB1R agonists Win 55212-2 and HU-210 reduce NMDA and AMPA-mediated EPSPs via a presynaptic mechanism [91]. These effects are mediated by CB1R activation since they were blocked by the CB1R antagonists AM258 and SR141716 [91].

A role for eCB signaling in the regulation of VTA dopamine neuron activity by inhibitory and excitatory inputs has been established [90]. In particular, increased VTA dopamine neuron excitability via blockade of postsynaptic, calcium-activated potassium channels (S_K) occludes Win 55212-2 inhibition of eIPSPs, suggesting that increased VTA dopamine neuronal activity activates eCB-mediated signaling. This concept is further supported by the finding that AM251 increases eIPSPs under these conditions, consistent with endogenous CB1R activation. Furthermore, presynaptic mGluR3 activation, which inhibits glutamate release, inhibits eCB-mediated signaling, suggesting that glutamate is the trigger for postsynaptic eCB synthesis/release. Conversely, when presynaptic mGluR3 receptors are blocked, glutamate release is augmented and eCB signaling is enhanced, as revealed by a large effect of AM251 to increase both IPSPs and EPSPs. The effects of mGluR3 inhibition and S_K channel blockade are additive, indicating that glutamate drives eCB synthesis through a mechanism other than increased neuronal activation in VTA dopaminergic neurons [90].

eCBs released under the stimulation conditions described above inhibit both inhibitory and excitatory inputs to VTA dopamine neurons, which supports CB1R expression by axon terminals of both excitatory and inhibitory afferent neurons [90]. Consistent with these findings, eCB signaling has been demonstrated to mediate DSE at excitatory synapses on VTA dopamine neurons [91]. A 10-s depolarization of VTA dopamine neurons results in a transient decrease in EPSP amplitude, an effect that was presynaptic in nature, mediated via CB1Rs, and dependent upon postsynaptic calcium rise. Activation of D2 receptors facilitates eCB-mediated DSE by enhancing eCB release [91]. These data suggest that, during VTA dopamine neuronal depolarization, somatodendritically released dopamine augments eCB synthesis via activation D2 autoreceptors, perhaps via augmentation of depolarization-induced intracellular calcium release. DSE is also elicited in VTA slices by brief trains of stimulations designed to mimic afferent input from the prefrontal cortex [92]. Using this induction protocol, CB1R-mediated suppression of excitation occurs and is likely mediated by 2-AG, since it is completely blocked by inhibitors of DAGL and partially blocked by inhibitors of PLC [92]. Afferent stimulation-induced suppression of excitation requires both intracellular calcium release and mGluR1 receptor activation, suggesting that both pathways are required for eCB synthesis/release in this stimulation protocol.

In an in vivo correlate of the slice study described above, Melis and colleagues found that stimulation of the prefrontal cortex results in phasic activation of VTA dopamine neurons [92]. Systemic administration of SR141716 enhances stimulation-induced VTA spiking activity, while Win 55212-2 decreases it, suggesting that activation of the VTA by the prefrontal cortex can induce eCB-mediated inhibition of excitation in vivo. Thus, CB1R-mediated signaling could play a role in shaping VTA dopamine neuron activity in response to activation by prefrontal cortical inputs. The functional implications of such a regulation are broad, and could have relevance to addiction, cannabis-induced psychosis, and the effects of cannabinoids on mood, affect, and motivation [93].

3.4 Striatum

The striatum is the primary input nucleus of the basal ganglia, receiving excitatory input from all neocortical regions, the thalamus, and BLA. Striatal neuronal projections go to the internal segment of the globus pallidus via direct and indirect pathways. The globus pallidal output projects to the thalamus, which in turn completes the cortico–striatal–pallidal–thalamic loop via projections back to the neocortex. Importantly, the striatum receives a dense innervation from midbrain dopamine neurons that modulates information flow from the cortex to the basal ganglia output nuclei, likely via alterations in the signal-to-noise ratio of incoming cortical excitatory inputs. Thus dopaminergic receptor activity can *select* specific, high-efficacy cortical inputs to be sent through to the output nuclei, while dampening weak, low efficacy cortical afferents (see [94] for review). This dopaminergic influence on striatal activity is markedly observed in patients with Parkinson's disease, in whom dopaminergic neurons have degenerated, resulting in movement disorders including hypokinesia, bradykinesia, tremor, and rigidity. The striatum is also involved in stimulus-response learning and the formation of habits that occur with overtraining. Thus, it has been suggested that the striatal neurotransmission contributes to habitual use of rewarding drugs [95].

The striatum expresses moderate CB1R density [5–7, 96, 97]. In general, CB1R expression is most robust in the lateral striatum and is lower in the central and medial regions [97]. CB1R are located on axon terminals of GABAergic neurons, which are likely axon collaterals of medium spiny neurons (the most abundant type of neuron within the striatum). These terminals form symmetric synaptic contacts onto cell bodies and dendrites of other medium spiny neurons [97]. Although these authors did not find CB1R expression on excitatory axon terminals [97], several other investigators have demonstrated CB1R expression on glutamatergic terminals within the striatal complex [98–100]. CB1Rs are not expressed by dopaminergic terminals within the striatum [101].

Consistent with its localization to glutamatergic and GABAergic nerve terminals, activation of CB1R has been demonstrated to modulate both afferent glutamatergic and GABAergic inputs to striatal neurons. Szabo and co-workers demonstrated that the CB1R agonists Win 55212-2 and CP55940 decrease eIPSPs recorded from medium spiny neurons of the striatum [102]. These effects are reversed by SR141716 and are likely presynaptic in origin since Win 55212-2 does not alter muscimol-induced IPSPs. These data suggest that CB1R activation modulates GABAergic transmission onto medium spiny neurons, likely originating from axon collaterals from other medium spiny neurons [102]. CB1R modulation of afferent glutamatergic transmission has also been demonstrated by several investigators. Win 55212-2 and HU210 decrease eEPSPs recorded from medium spiny neurons and increased the PPR by an SR141716-sensitive mechanism [103, 104]. Consistent with CB1R activation, the effect of Win 55212-2 is mediated by $G_{\alpha i/o}$ signaling and dependent upon N-type calcium-channel availability.

The effect of CB1R activation to reduce afferent glutamatergic transmission suggests a role for eCB signaling in the modulation of cortico-striatal synaptic transmission. In support of this hypothesis, HFS of cortical afferents to the striatum induce LTD of glutamatergic transmission, an effect absent in CB1R$^{-/-}$ mice and mice treated with the CB1R antagonist SR141716 [105]. Interestingly, SR141716 application 10 min after HFS does not block LTD, which suggests that eCB signaling is required for the induction of LTD but not its long-term maintenance [106]. LTD at this synapse is dependent upon increases in intracellular calcium and LTD is rescued in calcium-chelated cells by bath application of the eCB transport/degradation inhibitor AM404, suggesting that *spillover* of eCBs from nearby cells occurs [105]. Interestingly, intracellular application of eCB transport inhibitors blocks LTD induction by HFS, suggesting that a carrier-dependent mechanism is required for eCB efflux from neurons [106].

DSE also occurs at excitatory synapses onto medium spiny neurons of the striatum, although depolarization alone is not sufficient to induce eCB-mediated depression of EPSPs; coapplication of a mGluR1 agonist is required [107]. On the other hand, activation of mGluR1 alone does produce CB1R-dependent presynaptic inhibition of glutamatergic transmission [107]. Therefore, as in other brain regions, cooperative eCB synthesis by depolarization and mGluR1 activation exists in striatum.

Under what physiological conditions does striatal eCB signaling become engaged? Calabresi and co-workers explored striatal eCB signaling in an animal model of Parkinson's disease and found that depletion of striatal dopamine produces an increase in striatal AEA content [108]. An increase in cortico-striatal glutamatergic transmission also occurs, which is thought to contribute to the symptoms of Parkinson's disease (see references in [108]). These authors suggest that upregulation of striatal eCB signaling after dopamine depletion could represent a compensatory mechanism aimed at counteracting or normalizing the increased cortico-striatal glutamatergic transmission observed under these conditions. In support of this hypothesis, increased eCB/CB1R signaling via inhibition of AEA inactivation or direct CB1R activation reduces cortico-striatal glutamatergic transmission [108]. These authors also suggest enhancing eCB signaling could be useful in the treatment of Parkinsonian symptoms [108].

4 Conclusions

A picture is emerging from multiple experimental approaches that activation of CB1R on GABAergic and glutamatergic terminals results in inhibition of neurotransmitter release in many brain regions. The endogenous innervation of this receptor is accomplished by at least two arachidonic acid analogs, AEA and 2-AG. The source of the eCBs is postsynaptic neurons; thus, presynaptic CB1R activity is regulated by a retrograde synaptic signal. Direct and indirect evidence suggests that eCB mobilization is triggered by processes that

accompany or regulate postsynaptic neuronal activity. For example, depolarization, increased neuronal excitability, and metabotropic glutamate receptor activation all can induce eCB-mediated signaling. Interestingly, while the mechanism by which CB1R activation results in inhibition of synaptic activity is essentially identical in the brain regions investigated thus far, the mechanisms by which eCBs are mobilized are more diverse. Some of this diversity likely arises from the different signaling cascades involved in 2-AG and AEA biosynthesis, but studies of the kinetics of eCB signaling suggest that synthesis and release are separate processes that are regulated by different factors.

Acknowledgments The authors were supported during the writing of this review by the following National Institutes of Health grants: R01 DA09155, R01 DA16967, R01 NS41314, and F30 DA15575.

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New Insights into the Endocannabinoid System by Using Cannabinoid Receptor Knockout Mice

Meliha Karsak, Itai Bab, and Andreas Zimmer

Abstract Considering the profound effects of cannabinoids on the brain, it is perhaps not surprising that much of the efforts in the endocannabinoid field have been devoted to the elucidation of the function of the neuronal CB1 cannabinoid receptor and its ligands. Recent findings, however, have revealed some unexpected new roles of cannabinoid receptors in non-neuronal tissues such as the liver and bone and, consequently, the whole field has experienced a dramatic expansion. This chapter will to a large extend focus on these latest results obtained using cannabinoid receptor knockout mice.

Keywords Mice · Osteoporosis · Neurodegeneration · Animal models

1 Generation of the Knockout Mice

Like many other G-protein coupled receptors (GPCRs), the cannabinoid receptors are encoded by a single large exon. The murine CB1 receptor is encoded by the *Cnr1* and the CB2 receptor by the *Cnr2* gene, both of which are located on mouse chromosome 4. To date four lines of CB1 knockout mice have been established independently in different laboratories (Fig. 1). In the line generated by Ledent and co-workers [1], the first 233 codons were replaced by a PGK-neo cassette. Our laboratory generated a knockout line by replacing the region between amino acids 32 and 448 with PGK-neo [2]. Manzone and colleagues have replaced the first 356 amino acids [3]. Both mutations constitutively invalidate the gene, i.e. there is no residual gene function. CB1 receptor binding studies performed with our strain showed a complete lack of neuronal CP55,940 binding sites, and neither CP55,940 nor HU-210, nor THC stimulated any [^{35}S]GTP binding in the brains of homozygous mutant animals. However, there was still

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_11,

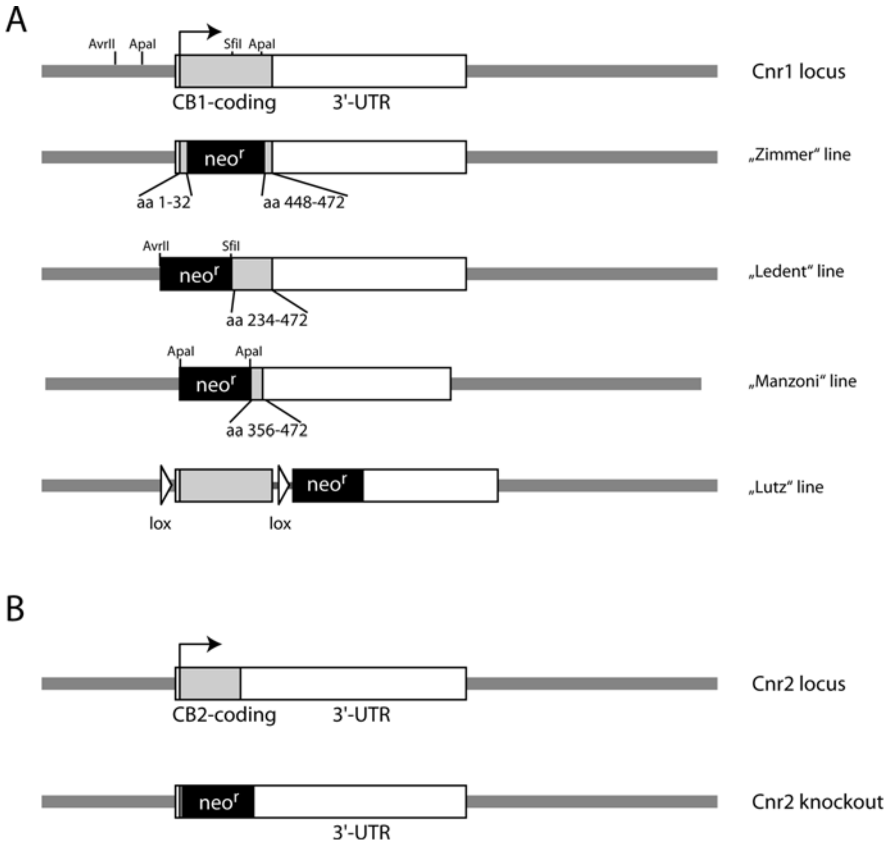

Fig. 1 Schematic representation of cannabinoid receptor genes and their allelic variants generated by homologous recombination. (**a**) The *Cnr1* locus encodes for the cannabinoid CB1 receptor in a single coding exon (gray box), which also contains a long 3′-untranslated region (white box). The knockout mouse lines generated by the Zimmer, Ledent, and Manzoni labs have all various regions of the coding sequence replaced by PGK-neo and thus represent null alleles. The mouse line generated by Lutz has two lox sites (triangles) flanking the coding region. Cre-mediated recombination between these lox sites consequently removes the entire coding region, upon mating to a Cre recombinase-expressing mouse strain. (**b**) In the knockout *Cnr2* line generated by our laboratory, part of the CB2 gene has been replaced by a PGK-neo gene, which is schematically not in scale depicted in figure 1 like described in detail in Buckley et al. (2000) [6]

some residual stimulation of [^{35}S]GTP binding by WIN55,212-2 in the brain, thus suggesting that this compound also binds to non-CB1 receptors.

The Ledent line has been crossed to the CD1 genetic background. Because CD1 is an outbred strain, it is likely that a unique substrain carrying the CB1 mutation has been generated during the breeding process. Similarly, the mutant mice generated by the Manzoni lab were crossed to C57BL/6 animals and heterozygous mice were then used to breed homozygous mutant animals [3].

The unique genetic composition of these lines makes it thus difficult to compare these animals with other knockout mouse strains, or with inbred strains of mice. Our mutant animals have now been crossed for more than 10 generations to C57BL/6 J mice, thus generating a congenic strain on this genetic background. Marsicano and colleagues [4] generated a third line of mice that carry a CB1 gene flanked by lox sites (*floxed*). These lox sites are recognized by the Cre enzyme, a DNA recombinase derived from P1 bacteriophages. When such mice are bred to transgenic line that expresses Cre, floxed genes are deleted in all tissues in which the Cre enzyme is active. This strategy is now frequently used for the tissue-specific inactivation of genes [5]. All CB1-deficient mouse lines are collectively referred to as *Cnr1*$^{-/-}$ mice throughout the text.

Our laboratory has also generated a CB2 knockout mouse strain (*Cnr2*$^{-/-}$ mice) [6], in which 341 base pairs of the *Cnr2* coding region were replaced by a neo^{r} cassette. These animals have also been backcrossed to C57BL/6 J mice to obtain a congenic *Cnr2* knockout line on a C57BL/6 J genetic background. Binding studies with spleen tissues showed an almost complete lack of CP55,940 binding in mice lacking CB2 receptors, indicating that this is the main cannabinoid receptor expressed by immune cells.

Interestingly, there are some residual cannabinoid effects in cannabinoid receptor-deficient mice. For example, the endocannabinoid 2-arachidonoylglycerol (2-AG) produced an interferon-gamma suppression in phorbol ester/ionomycin-activated mouse splenocytes isolated from CB1/CB2 double knockout mice [7]. In capsaicin-sensitive perivascular sensory nerves Zygmunt et al. found a THC and cannabinol induction of calcitonin gene-related peptide, which was CB1/CB2 cannabinoid receptor-independent [8]. And finally, hypotension was induced by the neurobehaviorally inactive abnormal cannabidiol in mice lacking both CB1 and CB2 receptors, and surprisingly this effect was blocked by cannabidiol and SR141716A [9].

2 Neuronal Deficits in CB1 Receptor Knockouts

The lack of CB1 receptors has severe deleterious consequences. Examination of the survival rates of *Cnr1*$^{+/+}$, *Cnr1*$^{+/-}$, and *Cnr1*$^{-/-}$ animals revealed a strikingly increased mortality rate in the absence of CB1 receptors [2]. At 6 months of age, over 30% of *Cnr1*$^{-/-}$ animal had died of natural causes, while less than 5% of the *Cnr1*$^{+/+}$ or *Cnr1*$^{+/-}$ mice had died by this age. Although the cause of death has not been elucidated conclusively, it may be related to the increased incidence of epileptic seizures in *Cnr1*$^{-/-}$ animals (our unpublished results) and their dramatically reduced threshold for kainic acid (KA)-induced seizures. After injection of 30 mg/kg KA, more than 75% of the *Cnr1*$^{-/-}$ mice, but less than 20% of *Cnr1*$^{+/+}$ animals died within 1 h of the injection [10]. The *Cnr1*$^{-/-}$ mice that survived the KA challenge showed significantly increased neuronal damage compared to *Cnr1*$^{+/+}$ animals when analyzed 4 d after the treatment.

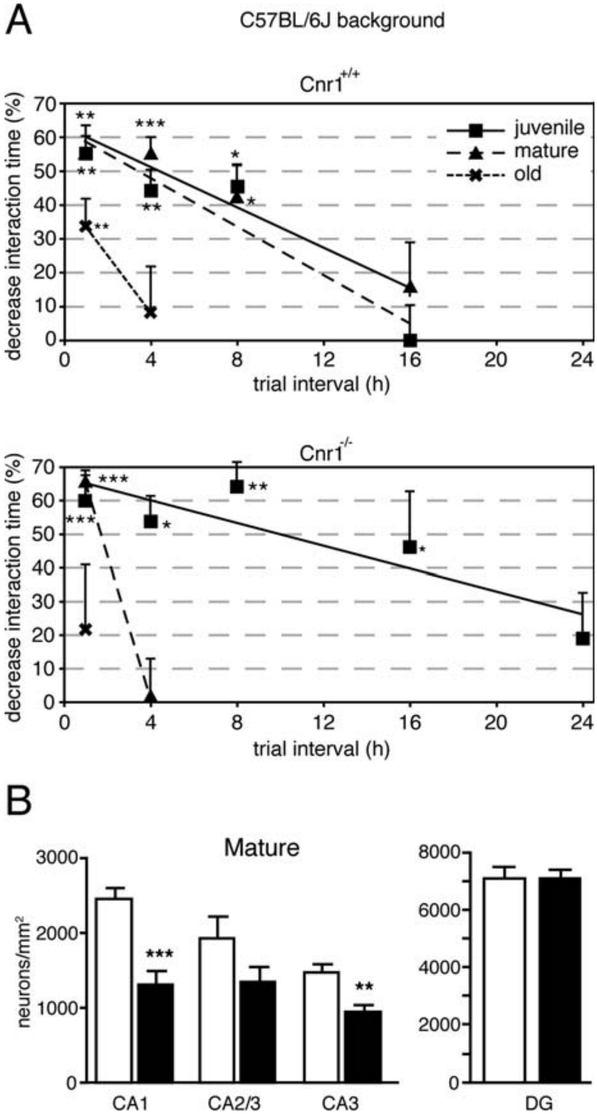

Fig. 2 Cognitive impairment and neuronal cell loss in *Cnr1*$^{-/-}$ mice. (**a**) In the partner recognition test, a reduction in the duration of exploratory social contacts between the first and subsequent trials indicated that the animal recognized the previously seen partner. Young and mature wild-type animals performed similarly in this test and readily recognized the previously seen partner after 8 h. In contrast, mature *Cnr1*$^{-/-}$ mice performed much worse and failed to recognize the partner after a 4-h interval. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ first versus second presentation. (**b**) The learning deficits are accompanied by a striking neuronal cell loss in the hippocampal CA1 and CA3 regions. The neuronal loss seemed to progress with age. Columns represent mean neuronal density expressed as number of neurons/mm^2 (±SEM); *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; *Cnr1*$^{-/-}$ versus *Cnr1*$^{+/+}$ mice. Modified after Bilkei-Gorzo et al. (2005) [14]

This result is consistent with the proposed CB1-dependent neuroprotective effects of endocannabinoids [11–13].

More recently, our laboratory has shown that *Cnr1* knockout mice have a stronger age-related decline of learning and memory functions [14]. While young *Cnr1*$^{-/-}$ mice (between 6–8 weeks of age) performed better than *Cnr1*$^{+/+}$ animals in several learning paradigms, mature *Cnr1*$^{-/-}$ animals (3–5 months old) showed dramatic memory impairments and performed in most tests at the level of old (14–17 months) animals (Fig. 2). For example, skill-learning abilities were evaluated in the rotarod test. Young *Cnr1*$^{+/+}$ mice needed on average 3.8 trials and mature animals 2.5 trials to reach their half-maximal performance, while old control mice required 7.1 trials and were rarely able to stay on the rod for 90 s.

In contrast, young *Cnr1*$^{-/-}$ mice needed only 2.7 trials. However, mature *Cnr1*$^{-/-}$ animals performed in this test just as badly as old animals. They required 5.3 trials to reach their half-maximal performance, and were not able to balance on the rotating rod for the 90 s duration of the test. An even more dramatic result was obtained in the social recognition test that assesses the ability of a mouse to remember a juvenile con-species mouse, which is presented on two consecutive sessions. Young and mature *Cnr1*$^{+/+}$ mice were able to recognize their partners 1, 4, and 8 h after the first exposure but not after 16 h. Again, young *Cnr1*$^{-/-}$ mice showed a better performance and still recognized their partners after 16 h. In stark contrast, mature *Cnr1*$^{-/-}$ animals only remembered their partners after 1 h, but not after a longer time interval. The old knockout mice were unable to recognize their partners even after 1 h. We performed these experiments originally with our mice generated on a C57BL/6 J background, but in collaboration with Olga Valverde we were able to reproduce these findings in the mouse strain generated by Ledent and co-workers [14]. Importantly, *Cnr1*$^{-/-}$ mice showed an accelerated age-dependent loss of hippocampal neurons, thus corroborating the behavioral findings. Neuronal cell counts in the hippocampus after Neu-N staining demonstrated a reduction in cell number in the CA1 and CA3 regions in *Cnr1*$^{-/-}$ compared to *Cnr1*$^{+/+}$ mice, which became more severe as the animals grew older.

3 The Cannabinoid System and Reward

There is an ongoing controversy among scientists, physicians, and in the general public about the addictive properties of cannabinoids, and to what extent cannabis consumption may contribute to the abuse liability of other drugs. While this controversy cannot be solved by animal experimentation, clearly animal models of drug addiction provide very valuable insights into the neurobiology of drug addiction, and experiments with CB1-deficient animal models have shown that the endocannabinoid system is an important modulator of the rewarding and addictive properties of other drugs of abuse.

Most of the common neurobehavioral and neurochemical effects of drugs of abuse are fulfilled by cannabinoids: they have rewarding properties as evaluated in conditioned place preference and intravenous self-administration paradigms, they stimulate the release of dopamine in the nucleus accumbens and somatic symptoms of cannabinoid withdrawal have been characterized in different animal species [15, 16]. The reinforcing and addictive properties of cannabinoids are clearly mediated by CB1 receptors, because *Cnr1*$^{-/-}$ mice failed to self-administer WIN55,212-2. Treating these mice chronically with THC did not evoke any symptoms of SR141716A-induced withdrawal.

The interactions between cannabinoids and other drugs of abuse have been studied using *Cnr1*$^{-/-}$ mice for opioids, nicotine, and alcohol. CB1 receptors evidently modulate the reinforcing and addictive properties of opioids, as morphine-induced place preference [17], morphine intravenous self-administration [1, 18], morphine-induced dopamine release in the nucleus accumbens [19], and morphine withdrawal syndromes were reduced or abolished in *Cnr1*$^{-/-}$ mice [1]. Interestingly, the interaction between the endogenous cannabinoid and opioid systems seems to be reciprocal. Thus, the rewarding properties of THC are reduced in μ-opioid receptor knockouts [20], while the dysphoric effects of THC are abolished in dynorphin-deficient mice [21] and κ-opioid receptor knockouts [22]. A decrease in the severity of the cannabinoid withdrawal syndrome was also observed in mice lacking both μ- and δ-opioid receptors [23].

There is also a substantial body of evidence for the involvement of CB1 receptors in the addictive properties of ethanol [24]. Ethanol treatment causes an increase in endocannabinoid synthesis and CB1 receptors are downregulated after chronic exposure to ethanol [25]. Analysis of the ethanol drinking behaviors using the two-bottle choice paradigm showed that the pharmacological blockade of CB1 receptors or their genetic deletion reduced ethanol consumption and preference [26–28]. However, this genotype effect seemed to be restricted to young mice, because older *Cnr1*$^{-/-}$ and *Cnr1*$^{+/+}$ animals showed a similar preference for ethanol [29]. The genotype effect was also influenced by stress levels [30]. When we analyzed *Cnr1*$^{-/-}$ and *Cnr1*$^{+/+}$ mice that were housed in a very quiet environment, there was no difference between the two genotypes, presumably due to reduced ethanol consumption by wild-type animals under low-stress conditions. Indeed, when *Cnr1*$^{-/-}$ and *Cnr1*$^{+/+}$ mice were exposed to a mild food-shock stressor, we found a significant increase in ethanol consumption in the mutant but not in the wild-type animals.

Together, these results demonstrate that endocannabinoid-modulation of ethanol preference is influenced by age and the environment. Most interestingly, withdrawal symptoms after the cessation of chronic ethanol exposure were completely absent in *Cnr1*$^{-/-}$ mice [30]. This finding is in line with a clinical study that demonstrated an association of a *CNR1* polymorphism with the severity of alcohol withdrawal symptoms in humans [31]. Hence, the endocannabinoid system may also play an important role in the development of alcoholism in humans.

The consumption of cannabis and tobacco is often associated. There is evidence that nicotine enhances some of the addiction-related properties of THC, as the co-administration of subthreshold doses of nicotine and THC produced a conditioned place preference [32]. Furthermore, co-administration of THC and nicotine attenuated the development of THC tolerance and enhanced the severity of SR141716A-induced cannabinoid withdrawal symptoms [32]. However, analysis of the nicotine behavioral responses in *Cnr1*$^{-/-}$ mice did not support a major role of the endocannabinoid system in the modulation of nicotine effects. Although nicotine-induced place preference was abolished in *Cnr1*$^{-/-}$ animals [33], there was no difference between knockout and wild-type animals in nicotine self-administration [18], nor in the severity of the nicotine withdrawal syndrome.

4 Impaired Oviductal Transport of *Cnr1*$^{-/-}$ Embryos

Cannabinoids have apparent adverse effects on embryonic development and pregnancy. The mechanisms for these effects, such as retarded embryo development, fetal loss, pregnancy failure, and reduced fertilizing capacity of sperm are not known [34–36], but involvement of the cannabinoid receptors is suggested by their expression in the pre- and postimplantation embryos [37–40]. In addition, the endocannabinoid synthesizing enzyme N-acyl-phosphatidylethanolamine-hydrolizing phospholipase D (NAPE-PLD) is expressed in the oviduct [41, 42], and a high level of anandamide is present in the uterus and oviduct [43]. Actually, the uterine anandamide level is even higher than that of the brain. CB1, but not CB2, receptors are also expressed in the oviducts on day 1–4 of pregnancy [42].

It was recently shown that defective CB1 receptor signaling leads to an impairment of the oviductal transport of embryos and to a concomitant pregnancy loss of about 40% [42, 44]. Ovulation and fertilization proceed normally in the absence of cannabinoid receptors in *Cnr1*$^{-/-}$ *Cnr2*$^{-/-}$, and *Cnr1*$^{-/-}$/*Cnr2*$^{-/-}$ mice. However, *Cnr1*$^{-/-}$ mice showed an asynchronous preimplantation development. On the morning on day 4 of pregnancy, when blastocysts normally implant into the uterus, only 64% of *Cnr1*$^{-/-}$ embryos were in the blastocyst stage, with a large number of embryos still being in the morula stage. Wild-type females normally have around 85% blastocysts on day 4. When *Cnr1*$^{-/-}$ females were mated to *Cnr1*$^{+/+}$ males, they delivered heterozygous embryos that were comparable in numbers to wild-type births, but 36% of the mutant females did not become pregnant at all. These results indicated that CB1 is important for normal embryonic development and that maternal CB1 deficiency leads to pregnancy failure. Using reciprocal embryo transfers Dey and colleagues showed that this failure is due to impaired embryonic transport in the oviduct, caused by the absence of CB1 but not CB2 [42]. Furthermore, when CB-specific antagonists and agonists were administered by miniosmotic pumps between days 1 and 4 of

pregnancy approximately 70% of wild-type mice treated with the CB1-specific antagonist SR 141716 showed an oviductal retention of embryos with asynchronous development whereas the CB2-specific antagonist SR 144528 had no effect. *Cnr1*$^{-/-}$ mice treated in the same way also showed a retention rate of 33%, which was similar to the nontreated mutant mice. These results show that the blockade of CB1 signaling leads to the observed phenotype and that acute pharmacological blockade of CB1 receptors had a stronger effect than permanent genetic invalidation of the *Cnr1* gene. Surprisingly, a similar oviductal retention of embryo transport was observed when an anandamide analog was administered [45]. This result was interpreted to suggest that any disturbance of an optimal endocannabinoid tone during normal pregnancy would adversely affect pregnancy rates. This idea is in agreement with a study that demonstrated biphasic effects of anandamide on blastocyst implantation [38].

The endocannabinoid system seems to affect not only embryo development, but also the oviductal transport of preimplantation embryos [44]. Rising progesterone levels during pregnancy lead to an increase in the sensitivity of noradrenaline to beta-adrenergic receptors (β-AR) at the circular muscle of the oviduct isthmus. Neuronal noradrenaline release results in muscle relaxation and thus facilitates the embryonic transport through the oviduct. CB1 receptors are expressed in the oviductal muscularis and they are coexpressed with β2-AR at the isthmus region. The comparison of the noradrenaline release of the *Cnr1*$^{-/-}$, *Cnr2*$^{-/-}$, and wild-type oviducts after challenge with increasing potassium chloride showed that only *Cnr1*$^{-/-}$ oviducts had a 33% enhanced [^{3}H]-noradrenaline overflow. This overflow was not inhibited by methanandamide treatment and it was also unaffected by the antagonist treatment. By contrast, in *Cnr2*$^{-/-}$ and wild-type mice the agonist treatment caused a 40% reduction of the [^{3}H]-noradrenaline concentration. Treatment with the CB1 antagonist SR 141716, but not the CB2 antagonist SR 144528, produced a [^{3}H]-noradrenaline overflow of 37% in wild-type oviducts. These results suggest that noradrenaline release is increased in the absence of CB1 receptors in the sympathetic nerve terminals, probably leading to alterations in the muscular tone. Because the embryo transport through the oviduct is caused by a wave of regulated contraction and relaxation of the oviductal muscularis, any change in the muscular tone could impact on embryo transport. The asynchronous preimplantation *Cnr1*$^{-/-}$ phenotype was rescued by treatment with the β-AR agonist isoproterenol. In wild-type mice an embryonic retention in the oviduct was observed after treatment with an α1-AR agonist (phenylephrine) alone and in combination with a β2-AR agonist (butoxamine). Interestingly, the *Cnr1*$^{-/-}$ phenotype could not be rescued by progesterone or estrogen but the embryonic development of *Cnr1*$^{-/-}$ embryos could be restored by prostacyclin and prostaglandin E_2.

Altogether the findings using *Cnr1*$^{-/-}$ mice showed that the CB1, but not CB2, signaling is involved in embryonic development and oviductal embryo transport. For mouse breeders it could be of interest that the isoproterenol treatment of *Cnr1*$^{-/-}$ mice normalized embryo transport. Since the breeding of

this mouse strain is sometimes difficult, such a treatment during the very first days of the pregnancy may be beneficial.

5 Colitis in CB1 Knockout Mice

Several studies have demonstrated a role of the endocannabinoid system and CB1 receptors in the modulation of gut motility [46, 47] and its involvement in intestinal inflammation. The endocannabinoids anandamide and 2-arachidonoylglycerol (2-AG) are present in relatively high levels in the gut [47–49], but intraluminal administration of these endocannabinoids caused inflammation in the rat ileum [50]. Pharmacological results indicate that this effect is independent of cannabinoid receptors, but dependent on the TrpV1 vanilloid receptor.

Using an experimental model for intestinal inflammation produced by croton oil, Izzo and colleagues observed a CB1 receptor expression upregulation. CP55,940 and cannabinol treatment caused a delay in intestinal motility, which was blocked by the CB1 antagonist SR 141716 [46]. Lutz and co-workers used another model for inflammatory bowel disease that involved the intrarectal infusion of 2,4-dinitrobenzene sulfonic acid (DNBS) [51]. In this model, mice were sacrificed 3 d after the DNBS treatment and the size and number of ulcerative lesions and/or inflammation sites in the colon were scored. *Cnr1*$^{-/-}$ mice displayed stronger inflammation signs, with 2.2-fold higher macroscopic scores and an increased myeloperoxidase (MPO) activity than *Cnr1*$^{+/+}$ mice. The colons of DNBS-treated *Cnr1*$^{-/-}$ animals showed an extensive hemorrhagic necrosis and infiltration of neutrophils into the mucosa. CB1 receptors thus seem to be involved in the early stages of the inflammation process. Finally, when the CB1 receptor was blocked with SR141716 30 min before and during the DNBS-treatment the mice developed even more severe transmural colitis.

These findings raised the possibility that treatment with a CB1 receptor agonist might alleviate colitis symptoms. Indeed, administration of HU-210, a CB1 and CB2 agonist, 30 min before as well as 24 and 48 h after intrarectal DNBS administration markedly reduced the inflammation signs scored by macroscopic and MPO analysis. Mice with a genetic deletion of the anandamide-degrading enzyme fatty acid amide hydrolase (FAAH), which have higher anandamide levels, were also analyzed in this disease model. DNBS-treated FAAH$^{-/-}$ mice showed clearly reduced inflammation scores as compared to wild-type mice. These experiments demonstrated that cannabinoids have anti-inflammatory properties in this colitis model and suggest that CB1 receptor activation protects the colon against inflammation. Electrophysiological recordings of the colon circular smooth muscle of *Cnr1*$^{-/-}$ mice showed spontaneous action potentials 8 h after DNBS challenge. It is possible that these spontaneous action potentials, as a sign of increased smooth muscular excitability, are caused by disruption of the afferent limb of intrinsic motor reflexes [52].

6 CB2 and Its Antifibrogenic Action in the Liver

Cannabinoid receptors are important modulators of hepatic hypotension, which is induced by the endotoxemic status of patients suffering from liver cirrhosis [53]. Kunos and co-workers have shown that CB1 receptors are upregulated on vascular endothelial cells isolated from cirrhotic human livers and are involved in the vasodilated state of cirrhotic patients. Recently, Lotersztajn and colleagues discovered that peripheral CB2 receptors, which are not present in the healthy liver, are upregulated in human cirrhotic samples [54]. They found a strong CB2 immunostaining signal in spindle-shaped cells in fibrotic septa in human biopsy specimen. CB2 positive signals were identified in nonparenchymal, as well as in inflammatory and bile duct epithelial cells located along fibrotic septa. This signal was independent of the disease etiology. CB2 receptors were also present in cultured human hepatic myofibroblasts and in activated rat hepatic stellate cells. Both cell types accumulate during chronic liver injury [55, 56]. The functionality of CB2 receptors in human myofibroblasts was demonstrated by ^{35}S-GTPγS-binding assays using THC as well as the CB2-specific agonist, JWH-015.

Hepatic fibrosis is a scar formation-type response, involving inflammation and fibrogenesis. The disease is associated with synthesis of excessive amounts of extracellular matrix components [57] composed of highly cross-linked collagen [58]. This scar-like tissue accumulates at the expense of functional hepatic parenchyma, a process resulting in progressive loss of liver function. Myofibroblastic cells originating from activated hepatic stellate cells and hepatic myofibroblasts proliferate and accumulate during the fibrogenic process. The production of fibrogenic cytokines such as TGF-β and the expression of smooth muscle α-actin (α-SMA) are markers for both cell types [59]. Because the clearance of hepatic stellate cells by apoptosis may ameliorate the disease condition, molecules regulating this process represent a potential therapeutic target [58], similar to substances with an antiproliferative effect.

Platelet-derived growth factor (PDGF) is the most potent mitogen for hepatic stellate cells. In cell viability assays using human myofibroblasts stimulated with PDGF-BB as a mitogen, CB2-specific and nonspecific agonists such as JWH-015 and THC had potently inhibited DNA synthesis. The antiproliferative effect of THC was blocked specifically with the CB2-antagonist SR144528. Endocannabinoids were also effective in this assay, but their effect was not be blocked by SR144528, thus indicating that endocannabinoids can also act via a CB2-independent mechanism.

The antifibrotic role of CB2 receptors was confirmed in CB2 knockout mice. When wild-type and CB2-deficient mice were treated with carbon tetrachloride, an inducer of liver fibrosis [60], *Cnr2*$^{-/-}$ mice developed an enhanced fibrosis with more frequent bridging fibrosis, whereas *Cnr2*$^{+/+}$ mice showed only moderate liver fibrosis. The higher fibrosis scores in *Cnr2*$^{-/-}$ mice were accompanied by increased hepatic collagen content and α-smooth muscle actin mRNA.

A high concentration of THC led to apoptosis in human myofibroblasts and rat hepatic stellate cells. This effect could be demonstrated by a time-dependent activation of the apoptotic marker caspase-3. Similar results were obtained with methanandamide and 2-AG, which showed again an apoptotic effect, which was not inhibited by the CB2 antagonist.

Since oxidative stress is a major player in apoptosis, the influence of CB2 activation on oxidative stress was examined. As an apoptosis marker the intracellular reactive oxygen species (ROS) was assessed, and indeed micromolar concentration of THC dose-dependently increased the production of ROS, thus concluding that the apoptotic THC effect depends on intracellular oxidative stress.

Taken together, these results demonstrate that the CB2 receptor activation has an antifibrogenic role, which is produced by the growth inhibitory and apoptotic effects of CB2 agonists. Two signaling pathways are responsible for these beneficial effects: COX-2 induction is involved in the antiproliferative action and the intracellular oxidative stress in the apoptotic process. However, the mechanism related to antiproliferative and apoptotic effects of methanandamide and 2-AG is unclear [54, 61, 62]. Because the CB2 antagonist SR 144528 could not block their action, CB2-receptor signaling is probably not involved. Very recently, it was found that CB1 receptors are also involved in the process of liver fibrosis. Prof. Lotersztajn and colleagues published that similar to CB2 receptors, the expression of CB1 is also upregulated in cirrhotic human liver [62]. However, in line with the finding that cannabis use is an independent predictor of fibrosis progression during the course of chronic hepatitis C [63], the CB1 activation promoted the progression of fibrosis [62]. In a model of murine liver fibrogenesis, the pharmacological and genetic ablation of the CB1 receptor led to a decreased accumulation of hepatic myofibroblasts and a reduced hepatic content of the profibrogenic cytokine TGF-β. Treatment with the CB1 antagonist rimonabant (SR 141716A) additionally enhanced apoptosis and/or decreased proliferation of liver fibrogenic cells. In conclusion, the CB1 antagonism is a promising candidate for the treatment of chronic liver injuries.

7 CB2 Receptor Activation has an Anti-Artherosclerotic Property in Mice

CB2 receptors are also involved in another important and common disease, namely, artherosclerosis. This cardiovascular disorder is the primary cause of heart disease and stroke in developed countries [64]. Mach and colleagues discovered that CB2 receptor expression is upregulated in human coronary artherosclerotic lesions, whereas the receptor is not expressed in healthy tissues [65]. In a mouse model for artherosclerosis the authors obtained similar results with CB2 expression only in the diseased arteries.

A well-established mouse model for atherosclerosis involves the feeding of apolipoprotein E knockout (*ApoE*$^{-/-}$) mice with a high-cholesterol diet [66]. Such mice quickly accumulate artherosclerotic lesions and plaques in the aortic root and arch, which also express CB2, but not CB1 receptors. This expression was found in macrophages and T lymphocytes using co-staining for mac-3 and CD4. Administration of a low oral dose of THC (1 mg/kg) together with a high-cholesterol diet significantly reduced the progression of artheroscleric lesions. Higher doses of THC showed lower effects. The effective dosage resulted in a blood THC-concentration of 0.6 ng/ml, which is well below the concentration required for the psychoactive THC effects [67–70]. The THC treatment effectively reduced macrophage infiltration to the lesions site and the recruitment of leukocytes to vessel walls. THC also inhibited the migration of peritoneal macrophages induced by IFN-γ or TNF-α in an in vitro assay. The beneficial effects of THC were abolished by the CB2 antagonist SR144528 and were absent in *Cnr2*$^{-/-}$ mice thus indicating that they are mediated by CB2 receptors.

Because T cells play an important role in early artherosclerosis development and Th1 cells are the predominant activated T cells within the aortic lesion site [71, 72], the question was whether THC produces a Th1/Th2 imbalance. Therefore, proliferation responses and cytokine profiles were analyzed in lymphoid cells. Splenocytes from animals treated with the low THC dose showed a significantly reduced proliferative response and IFN-γ secretion. Cytokines were not detectable (IL-4, IL-12) or only modestly reduced (IL-10, TGF-β) by the treatment. Thus, THC treatment induced a shift in the Th1/Th2 balance by suppressing the Th1 response, which may account for some of the anti-inflammatory properties of THC in the artherosclerosis model.

CB2 receptor activation thus seems to inhibit the progression of artherosclerosis by the inhibition of proliferation, migration, and a reduction of the Th1-response. These effects are relevant for several pathogenic processes. Interestingly, in human tissues and mouse models, CB2 receptor expression is upregulated in the disease state, while no expression was observed in the healthy tissue. The results clearly demonstrate a protective role of CB2 receptor activation in different disease models.

8 Regulation of Bone Mass by the Endocannabinoid System

The vertebrate skeleton undergoes continuous remodeling consisting of the concerted action of bone-forming cells, osteoblasts, and bone-resorbing cells, osteoclasts. It has been demonstrated that bone formation is subject to central control mediated by sympathetic efferent fibers [73]. This mechanism is regulated by leptin [73, 74], which is also a regulator of central endocannabinoid synthesis [75]. These findings prompted us to study the potential involvement of cannabinoid receptors in the regulation of bone remodeling.

A survey of cannabinoid receptor expression in bone cells showed that CB2 mRNA is progressively expressed, in parallel with other osteoblastic markers, in bone marrow-derived stromal cells grown in osteogenic medium [76]. By contrast, CB1 expression was very low. Similarly, CB2, but not CB1, receptors were also expressed in bone marrow-derived osteoclasts. Immunohistochemical analysis confirmed these findings and demonstrated CB2 receptor expression in osteoblasts, osteocytes, and osteoclasts.

Most strikingly, $Cnr2^{-/-}$ mice have a marked age-related trabecular bone loss and cortical expansion, which is reminiscent of human osteoporosis (Fig. 3). The bone volume density and the trabecular number were almost 50% lower in 1-year-old male CB2 knockouts compared to age-matched wild-type animals. At the age of 8 weeks $Cnr2^{-/-}$ mice already showed a 40% increase in osteoclast numbers and a 20% higher bone formation and mineral apposition rate, thus demonstrating a high bone turnover, which is a hallmark of postmenopausal osteoporosis. The accelerated bone resorption in $Cnr2^{-/-}$ mice is not fully compensated by the increased bone formation rate, thus resulting in a net bone loss.

The CB2-specific agonist HU-308 stimulated the proliferation of primary calvarial osteoblasts obtained from wild-type newborn mice through a $G_{\mathrm{i/o}}$ protein-dependent mechanism. The mitogenic effect of HU-308 was accompanied by an increase of tissue nonspecific alkaline phosphatase activity and extracellular mineral deposition, which are markers for osteoblast activity. In contrast, the CB1 agonist noladin ether had no effect. These results suggest that CB2 signaling stimulates the expansion of the preosteoblastic cell pool in a cell-autonomous manner, independent of osteoclast activity. Conversely, HU-308 inhibited the proliferation and differentiation of monocytic cells undergoing osteoclastic differentiation, again in a cell-autonomous manner. These effects of HU-308 were not observed with cell culture systems obtained from $Cnr2^{-/-}$

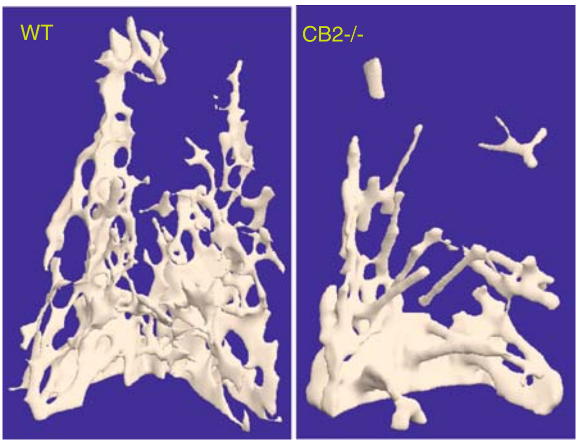

Fig. 3 Tridimensional microcomputed tomographic images of distal femoral metaphysis in 1-year-old wild-type (WT) and CB2-deficient mice (CB2−/−). The trabecular bone density and structure are markedly diminished in the absence of CB2. (*See* Color Plate 6)

mice. Thus, CB2 signaling is mitogenic in the osteoblast linage and antimitogenic in the monocytic–osteoclastic lineage.

Importantly, HU-308 treatment was also effective in female-ovariectomized (OVX) mice, an animal model of osteoporosis. When OVX mice were treated with HU-308, the trabecular bone loss was reduced to 27% in comparison to untreated mice which had a loss of 41%. The protective effect of HU-308 treatment was attributed to an attenuation of osteoclast-mediated bone resorption and a concomitant stimulation of osteoblast-mediated bone formation.

CB1 receptors have also been implicated in the regulation of bone formation, although CB1 receptors are not expressed, or only weakly expressed in bone. Ralston and colleagues described a high bone-mass phenotype in *Cnr1*$^{-/-}$ mice generated by Ledent. They also reported that CB1 knockout mice showed no significant bone loss after OVX [77]. In contrast, our male and female *Cnr1*$^{-/-}$ mice have a low bone-loss phenotype [78]. In order to resolve this discrepancy, we analyzed both mouse strains side-by-side using identical methods, equipment, and expertise. Interestingly, we observed a gender disparity in the Ledent mice, with male *Cnr1*$^{-/-}$ mice having a high bone mass and female *Cnr1*$^{-/-}$ mice having a normal trabecular bone with a slight cortical expansion [78]. The reason for the apparent phenotypic difference remains unclear, but may be related to the genetic background. However, we have not yet compared animals that were housed in the same facilities and therefore cannot exclude the possibility of environmental influences as well.

Our results obtained with the knockout mice then prompted us to look for a possible association between osteoporosis and polymorphisms in the human *CNR1* and *CNR2* loci. Indeed, we found a significant association of single polymorphisms and haplotypes encompassing the *CNR2* locus, but no convincing association with *CNR1* [79]. Together these results demonstrate an important function of the endocannabinoid system in the regulation of bone mass in mice and humans.

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Part V
Cannabinoid Receptor Pharmacology

Preclinical Pharmacological and Brain Bioassay Systems for CB1 Cannabinoid Receptors

Jenny L. Wiley and Billy R. Martin

Abstract Most psychoactive substances produce a broad array of in vitro and in vivo pharmacological effects in the central nervous system (CNS). Marijuana and its primary psychoactive substituent, Δ^9-tetrahydrocannabinol (Δ^9-THC), are no exceptions. Although many of these effects are mediated through the actions of cannabinoids on central cannabinoid CB1 receptors, the bioassay systems that have been used to assess these in vivo and in vitro effects of cannabinoids have varying degrees of pharmacological specificity. The purpose of this chapter is to describe and to evaluate the bioassays most commonly used to detect and characterize cannabinoid pharmacology. Although cannabinoids have been tested in many in vivo and in vitro procedures other than those described here, the focus of this review is on the bioassays that specifically target cannabinoids (particularly their abuse-related properties). The first half of the chapter will focus on in vivo models in which cannabinoids are active while the last half will concentrate on in vitro models.

Keywords Cannabinoids · CB1 receptor · Methods · Pharmacology

1 In Vivo Models

One of the fundamental tenets of pharmacology is that a drug cannot create new processes; it can only alter existing processes. In vivo models are based upon changes in an observable and measurable physiological or behavioral response or set of responses that occur when the drug is administered. Since the discovery of Δ^9-THC as the primary psychoactive substituent of marijuana [1], several in vivo bioassay systems have been developed to assess cannabinoid action. The results obtained with these assays, however, cannot speak directly to identification of

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_12,

underlying receptor mechanisms, as a combination of intracellular amplification steps and the interwoven nature of neuronal systems intervene in the translation from receptor activation to a subset of physiological responses that are possible within the species-limited repertoire. Hence, concomitant in vitro assessment is necessary for full evaluation of cannabinoid action. With these caveats in mind, however, in vivo models are, nonetheless, useful tools in characterizing and differentiating the various classes of cannabinoids.

1.1 Cannabinoid Tetrad

The *cannabinoid tetrad* is a battery of four tests that are sensitive to Δ^9-THC and other psychoactive cannabinoids [2]. Traditionally, these tests have been conducted in mice, but rats may also be used. The tetrad consists of measures of spontaneous activity, antinociception, rectal temperature, and catalepsy, all of which are measured in the same animal following drug injection. Spontaneous activity is assessed by placing the rodent in a chamber for a specified period of time and recording the frequency with which its movements break photocell beams across the chamber. Psychoactive cannabinoids dose-dependently decrease locomotion in this test. Maximal cannabinoid-induced inhibition of locomotion approximates 100%. Antinociception can be measured in several ways, but the most popular methods for the tetrad are tail flick and warm water tail withdrawal. In the tail flick procedure, the rodent's tail is placed under an intense heat source (e.g., a bright light) and latency for the rodent to flick its tail away from the heat source is recorded. Since this procedure may result in cutaneous damage to the tail (particularly when antinociceptive drugs are administered prior to the test), a maximum latency (10 s) is set, at which the heat source will be turned off by the experimenter. In addition, the ability to perform repeated testing with this type of model is limited (e.g., evaluation of tolerance or necessity of reusing scarce animal resource such as knockout mouse). Hence, when repeated testing is needed, the warm water tail withdrawal test is a better choice. In this test, the rodent's tail is placed in heated water and latency to remove it is recorded. As with the tail flick procedure, a maximum latency for tail removal is set. In addition, the temperature of the water can be varied for finer delineation of the antinociceptive effects of compounds. Cannabinoids increase the amount of time that rodent will expose its tail to the painful heat stimulus before moving it and is often expressed as percentage of maximum possible effect. The efficacy of Δ^9-THC for producing antinociception in the tail flick procedure is 100%. Cannabinoids also dose-dependently decrease body temperature, to a maximum drop of approximately -6°C. The final test in the tetrad is measurement of catalepsy. The traditional method of assessment involves placing the rodent on an elevated metal ring and measuring the amount of time during the entire session that it stays immobile on the ring. Catalepsy is dose-dependently increased by cannabinoids, with maximum effect

typically around 60% immobility in mice. In summary, then, the characteristic pharmacological effects of psychoactive cannabinoids in rodents are decreased locomotion, antinociception, hypothermia, and catalepsy.

The cannabinoid tetrad was developed during the period before CB1 receptors were cloned. Since traditional tetrahydrocannabinols and synthetic bicyclic cannabinoids were the only structural variations available at that time, this tetrad of tests was optimized for only a subset of cannabinoids. In the ensuing years, myriad cannabinoids of distinct structural classes have been discovered. For the traditional cannabinoids upon which the tetrad was normalized, binding affinity at CB1 cannabinoid receptors in the brain is strongly correlated with potency in each of the individual tetrad tests ($r = 0.85$–0.91) as well as with an average value calculated across tests [3]. Nonpsychoactive cannabinoids (e.g., cannabidiol) are not active in the tetrad nor do they bind to CB1 receptors [3].

Since the development of the tetrad test battery, other classes of cannabinoid agonists have been discovered – most notably, aminoalkylindoles and anandamides. While both of these novel classes of cannabinoids are also active in the tetrad, there are some notable differences. For example, whereas Δ^9-THC is approximately equipotent at producing each of the tetrad effects, aminoalkylindoles and indole-derived cannabinoids tend to be more potent at suppressing locomotion than at producing the other three effects [3, 4]. Conversely, anandamide and its analogs have approximately equal potency across measure; however, this class of compounds is less efficacious at inducing hypothermia (approximate maximum of –3°C) and often produces a greater maximum degree of catalepsy (e.g., 90%–95%) [5–7]. Despite the structural differences among the various classes of cannabinoids, correlations between CB1 binding affinities and in vivo potencies in the mouse tetrad remain strong for the plant-derived and synthetic tetrahydrocannabinols, bicyclic, and indole-derived cannabinoids [3, 4]. In contrast, correlation between these measures for anandamide analogs tends to be of lesser magnitude [8, 9], albeit the degree to which pharmacokinetic factors may contribute to this reduction has not been determined.

As noted above, structure–activity relationship studies have suggested that the effects of cannabinoids in the tetrad tests are mediated by CB1 receptor activation. Further support for this hypothesis is derived from challenge tests with the original selective CB1 receptor antagonist, SR141716A [10], and with novel analogs of this compound [11]. SR141716A dose-dependently reverses the effects of tetrahydrocannabinol and bicyclic cannabinoids as well as those of the aminoalkylindoles in the mouse tetrad [10, 12]. Interpretation of the results of antagonist tests with anandamide and anandamide analogs, however, is a bit more complicated. An issue of concern with respect to anandamide and other endogenous cannabinoids is the extent to which pharmacokinetics may play a role. Whereas traditional and bicyclic cannabinoids are metabolized slowly primarily through the hepatic P_{450} system [13], anandamide is rapidly hydrolyzed to arachidonic acid through the enzymatic action of fatty acid amide hydrolase (FAAH) [14, 15]. Adams et al. [16], for example, reported that

SR141716A did not block in vivo pharmacological effects of anandamide in the tetrad procedures in mice. Several reports suggest that anandamide's rapid metabolism almost certainly contributes to this lack of effect. First, while SR141716A did not block anandamide's effects in the tetrad, it did reverse the cannabimimetic effects of a more stable anandamide analog, 2-methyl-2′-fluoroethylanandamide in this model [16]. Second, SR141716A partially (but not completely) blocked the effects of anandamide in tetrad tests when its metabolism to arachidonic acid was inhibited by co-administration of phenylmethylsulfonyl fluoride (PMSF), a nonspecific amidase inhibitor that slows the metabolism of anandamide [17]. Finally, SR141716A reversed anandamide's pharmacological effects in the tetrad in FAAH knockout mice, but not in wild-type mice [18].

When administered alone, SR141716A is inactive in three of the tetrad tests: it does not produce antinociceptive, hypothermic, or cataleptic effects [12]. While it also does not suppress locomotor activity at active antagonist doses, SR141716A-induced locomotor stimulation has been observed [12]. Evaluation of the structure–activity relationship for this effect, however, suggests that the stimulatory activity of SR141716A and its pyrazole analogs is not mediated by a CB1 receptor mechanism [11, 19]. The CB2 cannabinoid receptor antagonist, SR144528, does not reverse the effects of cannabinoid agonists in the tetrad assays and does not produce effects of its own [20].

Although other classes of drugs (e.g., opioids) may produce one or more of the tetrad effects, the profile of tetrad effects is relatively selective for cannabinoids [2]. The battery of tests is *not*, however, pharmacologically *specific* and noncannabinoid compounds, including CNS depressants, antipsychotics, and transient receptor potential vanilloid type 1 ($TRPV_1$) agonists [21–23], may produce a similar profile of effects. Nevertheless, as reviewed above, results from these tests have been useful in characterizing the in vivo pharmacology of cannabinoids and have played a contributory role in determining the ways in which biochemical and receptor mechanisms are translated into pharmacological responses in live animals [24]. In addition, evaluation of antagonism by SR141716A increases the validity of these tests for prediction of CB1 receptor mediation, as the effects of these other classes of drugs are not altered by SR141716A [23].

1.2 Drug Discrimination

Drug discrimination is a more complicated and time-consuming assay of the CNS activity of certain psychoactive drugs that involves learning and repeated administration of the target drug. Despite these disadvantages, drug discrimination offers the distinct advantage of a degree of pharmacological selectivity that is unrivaled by other in vivo assays of cannabinoid action. The general training procedure for drug discrimination studies has utilized a two-lever

operant procedure involving the reinforcement (usually with food) of responses on one lever following administration of a specified (training) dose of Δ^9-THC (or another psychoactive cannabinoid) and reinforcing responses on a second lever following administration of vehicle. With consistent pairing of the type of injection and the position of the reinforced lever, animals can be trained over a number of days to respond reliably on the injection appropriate lever. In a well-trained animal, the percentage of total responses on the Δ^9-THC-associated lever approaches 90%–100% when the animal is given the training dose of Δ^9-THC and approaches 5%–10% when the animal is injected with vehicle (i.e., the animal responds on the vehicle-associated lever rather than on the Δ^9-THC-associated lever). An important distinction to note with regard to this procedure is that responding on the vehicle-associated lever does not imply that the administered drug does not have psychoactive or subjective properties of its own. Rather, responding primarily on the vehicle-associated lever indicates that any discriminative/subjective effects that the drug does have are not like those of Δ^9-THC (e.g., cocaine will produce a response almost exclusively on the vehicle-associated lever in a Δ^9-THC discrimination procedure).

After animals have acquired the discrimination successfully, substitution tests may be performed by injecting the animal with different doses of Δ^9-THC (in addition to the training dose) or with different drugs (to determine the sensitivity of the subject to Δ^9-THC or to determine the similarities of these novel agents to Δ^9-THC, respectively). Administration of other drugs of the same class or with different doses of the training drug produces a dose-dependent substitution pattern that, when graphed as a function of log dose, follows the sigmoidal shape of most dose–effect functions. Δ^9-THC antagonism studies are conducted by preadministering a receptor antagonist prior to evaluating the ability of subjects to detect Δ^9-THC during a test session. Common measures of the degree to which the substituted drug shares discriminative stimulus properties with Δ^9-THC are (1) percentage of responses on the Δ^9-THC lever or (2) percentage of animals that obtain their first reinforcer by pressing the Δ^9-THC-associated lever. Response rate (on both levers) is often presented as a secondary measure that provides information on general ability of the drug to disrupt responding (e.g., due to excessive sedation or motor stimulation or other behavioral factors). Generally, successful maintenance of the discrimination by training sessions on nontest days allows for substitution or antagonism testing of a number of compounds in the same group of animals.

Δ^9-THC discrimination is a pharmacologically selective procedure that has been validated as an animal model of marijuana intoxication [25]. Animals (including rats, gerbils, and nonhuman primates), predominantly press the Δ^9-THC-associated lever when injected with cannabinoids that humans say have marijuana-like subjective effects (for a review see [26]). In addition, potency for substituting for Δ^9-THC in this procedure is highly correlated with binding affinity for CB1 receptors [3, 4], suggesting that the discriminative stimulus effects are mediated by CB1 receptors in the brain. Conversely, non-cannabinoid drugs of many different classes (e.g., stimulants, antipsychotics,

antidepressants, and opioids) and cannabinoids that are not psychoactive (e.g., cannabidiol) do not engender responding on the Δ^9-THC-associated lever when administered to animals trained to discriminate Δ^9-THC from vehicle [27–29], although CNS depressants (particularly benzodiazepines) will produce some responding on the Δ^9-THC lever at high doses that also disrupt overall responding [30]. This partial responding on the Δ^9-THC lever does not seem to be CB1 mediated, however, as it is reversible by flumazenil but not by SR141716A [31]. Among cannabinoids with classical tricyclic structures similar to that of Δ^9-THC itself, dose-dependent full substitution (80%–100% responding on the Δ^9-THC-associated lever) occurs.

While most early drug discrimination studies examined the effects of classical tricyclic cannabinoids in Δ^9-THC discrimination procedure, more recent studies have demonstrated that bicyclic cannabinoids (e.g., CP 55,940) and aminoalkylindoles (e.g., WIN 55,212-2) also substitute fully for Δ^9-THC [32, 33] and can serve as training drugs themselves [34, 35]. In fact, previous studies have clearly shown that drugs from each of these major cannabinoid classes substitute and cross-substitute for each other, regardless of which cannabinoid (i.e., Δ^9-THC, CP 55,940, or WIN 55,212-2) is used as the training drug (for a review see [26]). Further, in both rats and rhesus monkeys, their potencies for doing so correspond with their binding affinities for CB1 receptors [4, 32, 33].

More definitive evidence that supports a primary role for CB1 receptor mediation of Δ^9-THC's discriminative stimulus effects derives from antagonist studies. SR141716A dose-dependently blocks the discriminative stimulus effects of three of the major classes of cannabinoids, as represented by prototypic drugs, Δ^9-THC, CP 55,940, and WIN 55,212-2 and it does so in several species, including rats, pigeons, and rhesus monkeys [34–37]. In contrast, drugs from a number of other pharmacological classes failed to block its discriminative stimulus effects [28]. Several cannabinoid compounds that have been tested as antagonists also failed to attenuate the discriminative stimulus effects of Δ^9-THC. Indeed, these compounds, $\Delta^{9,11}$-THC and cyano and nitrogen mustard-derivatives of Δ^8-THC, substituted for Δ^9-THC, albeit with different time courses and/or potencies [38, 39]. Together, these results clearly demonstrate that classical tricyclic cannabinoids, bicyclic cannabinoids, and aminoalkylindoles share discriminative stimulus effects and that these effects are mediated through the action of these drugs on CB1 cannabinoid receptors.

Results of substitution and cross-substitution tests with anandamide and anandamide analogs present a murkier picture. Although anandamide has been tested in cannabinoid discrimination paradigms in several labs, results are conflicting. Full substitution of anandamide for Δ^9-THC and CP 55,940 was observed in one study with rats, but only at doses that also decreased overall responding [40]. (In contrast, Δ^9-THC and the other classes of cannabinoids substitute and cross-substitute for each other at doses that have no effect on response rates, suggesting that there may be differences as well as similarities in the discriminative stimulus effects of anandamide and Δ^9-THC.) This original finding of substitution with anandamide in rats was not replicated in a

subsequent study in the same lab [41] or in different labs [42, 43] nor does anandamide substitute for Δ^9-THC in rhesus monkeys [44]. In addition, anandamide does not serve as a discriminative stimulus itself [41], probably because its rapid metabolism makes its stimulus properties unreliable.

Since these early studies, anandamide analogs with increased metabolic stability have been synthesized and tested in Δ^9-THC discrimination procedures. In structure–activity experiments with anandamide analogs in a discrimination paradigm in rats, Δ^9-THC-like discriminative stimulus effects did not occur with anandamide analogs in which the arachidonyl constituent was saturated or in which substitution was made for the ethanolamide constituent or C2' hydroxyl [41–43]. Interestingly, these compounds had little or no affinity for CB1 receptors when binding was assessed in the absence of PMSF to block fatty acid amide metabolism [8, 9]. In contrast, anandamide analogs in which a methyl was added to increase stability showed the greatest degree of substitution for Δ^9-THC in rats, albeit substitution was usually accompanied by suppression of response rates (as it was in a study in which anandamide substituted) [41, 45]. In rhesus monkeys, another methylated anandamide analog, 2-methylarachidonyl-2'-fluoroethylamide, fully substituted for Δ^9-THC in the absence of response rate effects [46]. Binding affinities of these methylated compounds measured with and without the enzyme inhibitor PMSF added to binding medium are more similar to each other than they are for analogs with other types of manipulations [8, 9].

Although the differences in patterns of substitution for Δ^9-THC observed with anandamide analogs versus those seen with other classes of cannabinoid agonists are relatively minor as compared with differences across drug classes or with differences among cannabinoids in other test batteries, attempts have been made to increase the selectivity of Δ^9-THC discrimination. Two procedural variations are noteworthy. In the first, the training dose of Δ^9-THC was manipulated. (R)-Methanandamide (AM-356) fully substituted for Δ^9-THC when the Δ^9-THC training dose was 1.8 or 3 mg/kg; however, only partial substitution of (R)-methanandamide was reported at a Δ^9-THC training dose of 5.6 mg/kg [43, 47], suggesting that manipulation of training dose may aid in distinctions among different classes of cannabinoids. A pattern of increased pharmacological selectivity with higher training doses has also been observed previously with other classes of discriminable drugs [48–50]. Interestingly, manipulation of the training dose of CP 55,940 did not affect maximal substitution produced by Δ^9-THC, suggesting subtle differences between the discriminative stimulus effects of anandamide-like analogs and those of other classes of cannabinoids. Based upon these results and results obtained from SR141716A antagonism tests in rats discriminating different doses of CP 55,940, De Vry and Jentzsch [51] suggest that manipulation of training dose may offer an in vivo method of estimating intrinsic activity of different cannabinoid agonists. A second procedural variation that has been used to increase selectivity of Δ^9-THC discrimination is to increase the number of drugs associated with the second lever (i.e., the one *not* associated with Δ^9-THC); hence, rather than discriminating

Δ^9-THC from vehicle, rats discriminated Δ^9-THC from a number of other compounds, including morphine and phencyclidine [52]. Rats injected with methanandamide responded primarily on the Δ^9-THC-associated lever when the other lever was associated with vehicle only; however, when the other lever was associated with vehicle and other drugs, response following methanandamide administration occurred on both levers, indicating greater selectivity for Δ^9-THC with this procedure than with the more traditional Δ^9-THC versus vehicle procedure.

Another approach to examining similarities and differences among the discriminative stimulus effects of anandamide analogs and those of other classes of cannabinoids is to train animals to discriminate an anandamide analog from vehicle. Although anandamide itself does not serve as an effective discriminative stimulus, two methylated analogs (R)-methanandamide (AM-356) and O-1812 [(*R*)-(20-cyano-16,16-dimethyl docosa-*cis*-5,8,11,14-tetraenoyl)-1′-hydroxy-2′-propylamine] have been trained as discriminative stimuli [45, 53]. In addition, Δ^9-THC produced full dose-dependent substitution in discriminations based upon each of these training drugs. Substitution of each anandamide analog for Δ^9-THC in Δ^9-THC-discriminating rats was also observed, as mentioned previously; however, this substitution was accompanied by decreases in response rates for most rats. In contrast, the $TRPV_1$ receptor agonist, O-1839, failed to substitute for O-1812 [45]. The fact that it produced effects similar to this drug in the tetrad tests in mice [21] highlights the greater degree of pharmacological specificity of cannabinoid discrimination as compared with other in vivo procedures for detecting cannabinoid activity. CB1 receptor mediation of the discriminative stimulus effects of (R)-methanandamide and O-1812 is indicated by the finding that CB1 antagonists, SR141716A and/or AM251, dose-dependently blocked the discriminative stimulus effects of these anandamide analogs whereas the CB2 antagonist, SR144528, did not [45, 54].

While the majority of drug discrimination studies with cannabinoids have focused on cannabinoid agonists as training drugs, there have been a few attempts to train animals to discriminate SR141716A from vehicle. Initial efforts that used two-lever appetitive-motivated operant procedures in rats and pigeons were unsuccessful [34, 37], suggesting that SR141716A did not have discriminative stimulus effects of its own. In two more recent studies, however, SR141716A was successfully trained as a discriminative stimulus using nonappetitive methods. Both alternative procedures involved the pairing of an aversive stimulus with SR141716A. Jarbe et al. [55] used discriminated taste aversion, a variant of traditional conditioned taste aversion procedures. In this procedure, two groups of water-restricted rats were allowed access to water during test sessions and the amount of water consumed was recorded. Prior to each training session, rats received an injection of SR141716A or an injection of vehicle. After each training session, rats in the control group received an injection of saline, regardless of the pretreatment injection. In contrast, rats in the experimental group were injected with saline only if they had received a presession injection of vehicle. On days when SR141716A injection (i.e., the training stimulus) preceded the training session, these rats were injected with

lithium chloride, a toxin that causes nausea and malaise. Consistent pairing of SR141716A with the aversive condition produced by the toxin resulted in decreased fluid consumption following SR141716A injection (but not following vehicle injection), suggesting that the rats were able to discriminate the stimulus effects of SR141716A and that these effects served as a *warning* of imminent feelings of nausea. Since rats in the control group drank approximately equal amounts of water regardless of preinjection of SR141716A or vehicle, direct pharmacological effects of SR141716A on fluid consumption were not responsible for the decreased drinking observed in the experimental rats. Further, substitution tests showed that this effect was dose-dependent and that the CB1 antagonist AM-251, but not the CB2 antagonist SR144528, also selectively decreased drinking in the experimental rats. Together, these results strongly suggest that SR141716A was serving as a discriminative stimulus. This procedure has been successfully employed previously in similar situations in which attempts to train animals to discriminate a drug (often an antagonist; e.g., naloxone) in an appetitive-motivated procedure failed [56].

The other successful attempt to train SR141716A as a discriminative stimulus occurred in rhesus monkeys [57]. This study differed from previous cannabinoid discrimination studies on a number of variables; however, arguably the two most crucial differences were the use of negative reinforcement and the use of a Δ^9-THC-dependence baseline, each described in more detail below. Whereas all of the cannabinoid agonist discrimination studies described thus far involved training food- or water-restricted animals to press a lever to receive food or water, respectively, McMahon and France [57] trained monkeys to press a lever to avoid receiving (or escape) a mild electric shock. On days that the monkeys received a presession injection of SR141716A, pressing on one of the levers would result in shock avoidance or termination. After vehicle injection, presses on the other lever were required. Hence, one way in which this study differed notably from previous studies was that negative reinforcement (shock termination), rather than positive reinforcement (food), was used to maintain responding. A second major difference in this study was that the monkeys were made dependent upon Δ^9-THC before discrimination training was initiated. In Δ^9-THC-dependent monkeys, an injection of SR141716A precipitates withdrawal whereas an injection of vehicle does not. Within the context of a discrimination paradigm, then, responses on the 'SR141716A' lever are associated with the stimulus effects of SR141716A-precipitated withdrawal rather than with the stimulus effects of the drug itself. Nevertheless, a degree of selectivity was demonstrated. Whereas SR141716A engendered responding on the SR141716A-associated lever in a dose-dependent manner in these monkeys, ketamine and cocaine did not. Further, SR141716A's discriminative stimulus effects were attenuated by Δ^9-THC. Finally, termination of Δ^9-THC administration also resulted in responding on the SR141716A-associated lever for most monkeys, but only after at least 24 h abstinence, suggesting that subjective effects of withdrawal following termination of the dependence regimen of

Δ^9-THC administration, although delayed, may be similar to those of precipitated withdrawal.

In summary, the results of drug discrimination studies have revealed remarkable similarities among the discriminative stimulus effects of classical and bicyclic cannabinoids and the aminoalkylindoles. Anandamide-like cannabinoids share some of the discriminative stimulus characteristics of these other classes of cannabinoids, in that substitution and cross-substitution occur in rats trained to discriminate either Δ^9-THC or an anandamide analog from vehicle. Some differences are also apparent, however, as anandamide analogs typically substitute for Δ^9-THC only at doses that reduce overall responding. In addition, manipulations designed to increase the selectivity of Δ^9-THC discrimination result in less substitution by methanandamide, but do not affect the maximal substitution produced by Δ^9-THC or CP 55,940. Nevertheless, the discriminative stimulus effects of the four major classes of cannabinoid agonists (tetrahydrocannabinols, bicyclic cannabinoids, aminoalkylindoles, and anandamides) are blocked by SR141716A, suggesting CB1 receptor mediation.

1.3 Self-Administration/Conditioned Place Preference

Self-administration is an animal model of the rewarding effects of drugs and is predictive of drugs that are voluntarily used by humans for their euphoric effects [58, 59]. The most commonly used version of self-administration is intravenous (I.V.) self-administration. In this model, the animal is implanted with a chronically indwelling catheter (usually in a jugular vein), through which drug can be administered I.V. When the animal emits a certain response (e.g., presses a lever), it receives an infusion of a set amount of drug through the catheter. The frequency with which the animal presses the lever to receive drug is a measure of its ability to serve as a reinforcer. Dose–effect functions, in which the administration dose (converted to log) is plotted against the number of infusions of the dose, typically take the form of an inverted U-shaped distribution; that is, doses in the middle of the reinforcing range will increase responding to a greater extent than lower doses or higher doses (which may be subthreshold or incapacitating, respectively). Within this basic paradigm, possible procedural variations are numerous, but several points are worth mentioning here. First, a second lever is often present in the self-administration chamber. Responses on this second lever may serve as a way to measure nonspecific effects of the drug that is being administered. In some cases, responses on the second lever do not produce any consequences for the animal. In other cases, responses may result in food delivery. Second, the baseline level of responding in a self-administration procedure (i.e., when the animal receives an infusion of vehicle for responding) is not usually zero. At the beginning of each session, an animal that has acquired the procedure usually samples the drug/vehicle, with responding dropping off as the session proceeds if the drug is

not reinforcing. Third, most self-administration procedures provide limited access to the drug; that is, lever presses deliver an infusion of drug only during certain times of the day (usually indicated to the animal by presence of an external stimulus such as a light). Unlimited access conditions (24-h a day and 7-d a week) may result in patterns of use that approximate those of humans to a greater extent, but are not always compatible with maintenance of the animal's health. Fourth, efficacy comparisons among drugs is not possible in the typical self-administration procedure that uses a fixed ratio schedule (i.e., a set number of responses results in an infusion of drug), although alterations in the schedule parameters may be made to allow for this type of comparison. Finally, it is important to note that self-administration and drug discrimination do not provide identical information and a drug may not be active in both procedures. For example, many antipsychotics have discriminative stimulus effects, but they are not self-administered [60, 61].

Only within the last decade have investigators been successful in training animals to self-administer cannabinoids (reviewed in [62]). An early study reported that rhesus monkeys trained to self-administer I.V. phencyclidine did not self-administer Δ^9-THC or CP 55,940 [63]. Since marijuana is consumed voluntarily by humans, cannabinoids were assumed to be *false negatives* in this preclinical predictive procedure. In 2000, however, Tanda et al. [64] reported success in training cocaine-experienced squirrel monkeys to self-administer Δ^9-THC. Subsequently, this group showed that Δ^9-THC was self-administered in drug-naïve squirrel monkeys [65] and that anandamide and methanandamide were also self-administered [66]. More recently, investigators have demonstrated self-administration of Δ^9-THC, CP 55,940, and WIN 55,212-2 in rats [67–69]. The reinforcing effects of each of these drugs were blocked by SR141716A [64, 66–69], suggesting mediation by CB1 receptors. SR141716A itself is not self-administered in monkeys [70].

Although the self-administration procedure has the greatest degree of validity for prediction of drugs that have reinforcing effects in humans, conditioned place preference has also been used as a preclinical method to assess rewarding effects of drugs. This type of procedure is conducted in a two- or three-chambered box, with each chamber separated from the other(s) by removable door(s). In addition, each compartment has environmental cues that distinguish it from the other compartment (e.g., white vs. black walls). Prior to training, a habituation period is instituted, during which the rodent is allowed to explore all of the chambers. Time spent in each compartment is usually recorded. During the ensuing acquisition (conditioning) phase of the experiment, the rat or mouse is injected with the target drug on some days and is confined to one of the two outside compartments for a set period of time. On other days, the rodent is injected with vehicle and is confined to the other outside compartment. The choice of which of the two outside chambers is paired with the drug and which with the vehicle may be biased or unbiased. A biased procedure uses information about where the rodent spent time during the habituation period to decide which chamber will be paired with the drug. In

most biased procedures, the drug is paired with the chamber that in which the rodent spent the least amount of time. An unbiased procedure indicates that the habituation period results did not influence the choice of which chamber to pair with the drug. Unbiased procedures may also result if the rodents prefer both chambers equally during the habituation period. In either case, after the rodent is exposed to a number of pairings between drug or vehicle and the respective chambers of the apparatus, a test session is conducted in which the doors separating the chambers are removed. At the beginning of this test session, the (drug-free) rodent is placed in between the two outside compartments (or in the third center compartment) and the amount of time spent in each compartment is recorded. If the rodent spends significantly more time in the compartment that was associated with the target drug during the training phase, conditioned place preference has developed whereas significantly less time in this compartment is indicative of conditioned place aversion.

The theory underlying conditioned place preference/aversion is Pavlovian or classical conditioning. In classical conditioning, an unconditioned stimulus (e.g., the target drug) elicits an unconditioned response (e.g., pleasure or aversion). If the unconditioned stimulus is repeatedly paired with another conditioned stimulus (e.g., a specific environment, as in one side of the conditioning chamber), an association between the conditioned stimulus and the unconditioned response develops. The net result is that the rodent will prefer exposure to the compartment that was associated with the drug if the drug produced pleasure (i.e., conditioned place preference) and will avoid the compartment if the drug exposure was unpleasant (i.e., conditioned place aversion). In contrast, self-administration is based upon operant learning theory, in which the frequency of lever pressing increases if this response is followed by delivery of a reinforcer (e.g., the target drug) or it decreases (extinguishes) if the response is not associated with a reinforcer. Although both of these procedures provide information concerning the hedonic value of the target drug, this information is not identical. The self-administration procedure assesses the ability of the drug to serve as a reinforcer through measurement of the frequency with which an animal will perform a response in order to receive an infusion. Conditioned place preference is an evaluation of the degree to which an association develops between the rewarding or aversive properties of the drug and the environment in which these properties were repeatedly experienced. Since the two procedures measure different constructs, results from one may not correspond in a predictable manner with those from the other; that is, drugs that are self-administered may not produce conditioned place preference and vice versa. A drug that is associated with pleasure when self-administered may not be associated with pleasure when it is administered by an experimenter.

Cannabinoids represent a class of drugs that produce seemingly disparate results in self-administration and conditioned place procedures. Whereas recent studies have clearly shown that Δ^9-THC and other cannabinoids are self-administered, results in conditioned place procedures are less consistent. Some studies have reported that Δ^9-THC and other psychoactive cannabinoid

agonists produce conditioned place preference [68, 71–73]. Most investigators, however, have found that cannabinoid agonists, including Δ^9-THC, CP 55,940, and WIN 55,212-2, produce conditioned place aversion [74–78]. Although myriad procedural variations among the studies complicate specification of factors that may contribute to the observed differences, most studies in which cannabinoid-induced place preference was demonstrated required training under special circumstances (e.g., pre-exposure to the drug or central administration). In studies where SR141716A was tested, it blocked cannabinoid-induced preference and aversion. In addition, a couple of studies found that SR141716A itself produces place preference [74, 78].

As mentioned, results of self-administration studies are not always consistent with those of conditioned place preference/aversion studies and cannabinoids provide an illustrative example. Marijuana is clearly self-administered by humans, albeit usually via smoking rather than I.V. injection. Under experimental conditions that mimic certain dosing and timing parameters of human self-administration, Δ^9-THC and other cannabinoid agonists are also self-administered by rats and by nonhuman primates. In contrast, most studies have reported Δ^9-THC-induced place aversion rather than place preference. An overriding conclusion of the combined data from both procedures is that preclinical evaluation of the reinforcing and reward-associated effects of cannabinoids is not as straightforward and reliable as it is for some other classes of abused drugs, including psychomotor stimulants, opioids, or CNS depressants.

1.4 Chronic Effects: Tolerance and Dependence

In humans, the effects of repeated or chronic use of cannabinoids are of much greater concern than their acute effects. Although the number of individuals that have tried marijuana once far exceeds the number of marijuana abusers, long-term abuse has the potential for a more negative impact on an individual as well as on society. The chronic effects of cannabinoids are also of interest with respect to their potential medicinal use. Because many of the putative therapeutic effects of cannabinoids are for chronic or continuing disorders, individuals conceivably would be taking these drugs repeatedly for an extended period of time. Hence, assessment of the effects of chronic dosing (especially with regard to the development of tolerance and dependence) is important.

Evaluation of in vivo tolerance development is relatively straightforward for the tetrad tests. Essentially, the effects of the inducing drug are measured after a period of repeated dosing. These effects are compared to those produced by acute dosing in rodents repeatedly treated with vehicle or to those assessed in an initial test in the same animals before the tolerance induction. Bass and Martin [79] have reported that maximal tolerance develops with supplemental injections over a period as short as 3.5 days. (Complications associated with

assessment of tolerance to the discriminative stimulus effects of cannabinoids are discussed in greater detail in [80] and will not be presented here.)

Numerous reviews from several different perspectives have been written on the topic of cannabinoid tolerance and dependence [81–84]. The general conclusion of these reviews is that many of the in vivo pharmacological effects of tetrahydrocannabinol cannabinoids that have been observed preclinically exhibit profound tolerance (up to 100-fold) and that this phenomenon occurs across species (for a review see [85]). Repeated dosing with bicyclic cannabinoids and aminoalkylindoles also produces dramatic tolerance to their own effects as well as cross-tolerance to those of Δ^9-THC [86, 87]. Since pharmacokinetic parameters (absorption, distribution, metabolism, and excretion) for tetrahydrocannabinol cannabinoids are relatively unaltered by chronic administration, pharmacodynamic factors are probably most responsible for tolerance development. This hypothesis receives further support from the finding that substantial CB1 receptor downregulation and reduced second messenger signaling occurs in the brains of Δ^9-THC-tolerant rodents [88, 89].

Tolerance to the effects of anandamide and its analogs in the tetrad tests also occurs, although the magnitude of this tolerance is often less than that produced by Δ^9-THC and other classical cannabinoids [90–93]. In addition, cross-tolerance between anandamide and Δ^9-THC develops for some of these effects under certain conditions, but not for all of them [87, 91, 93, 94]. Further, the magnitude of cross-tolerance between anandamide-like drugs and Δ^9-THC is dependent upon the test [95]. This task specificity suggests possible differences in the mechanisms through which the separate classes of cannabinoids produce their in vivo effects. Pharmacodynamic factors undoubtedly are involved as differences in cross-tolerance among anandamide-like and classical cannabinoids also occur in an isolated tissue preparation [87].

In addition to tolerance induction, repeated dosing with cannabinoids may also result in dependence. Dependence implies the appearance of symptoms of withdrawal when the drug is no longer available after a period of repeated administration. The drug can be made *unavailable* through abrupt termination of dosing (i.e., spontaneous withdrawal) or by injection with an antagonist (i.e., precipitated withdrawal). Symptoms of spontaneous withdrawal upon abrupt cessation following repeated cannabinoid administration are not readily apparent in most preclinical paradigms as it is for other classes of drugs such as CNS depressants and opioids. When reported, spontaneous withdrawal usually requires heroic doses or continuous infusion of the cannabinoid [96]. Hence, most studies of cannabinoid dependence have used precipitated withdrawal. In this approach, dependent animals that have been chronically treated with a psychoactive cannabinoid are administered a CB1 receptor antagonist such as SR141716A. Symptoms of SR141716A-precipitated withdrawal in rats and mice that have been treated chronically with Δ^9-THC or WIN 55,212-2 include head shakes, facial tremors, tongue rolling, biting, wet-dog shakes, eyelid ptosis, facial rubbing, paw treading, retropulsion, immobility, ear twitch, chewing, licking, stretching, and arched back [97–99]. Verification of these effects as

comprising a withdrawal syndrome was achieved through demonstration that Δ^9-THC readministration reversed the effects [99].

Development of dependence following chronic administration of anandamide is unclear. Whereas some investigators have observed SR141716A-precipitated withdrawal after repeated administration of anandamide [90], other researchers have found that SR141716A did not precipitate symptoms of withdrawal, even after extreme anandamide-dosing regimens [100]. Unreliability of withdrawal effects following anandamide administration is reminiscent of the inconsistent or distinct pattern of results produced by the class of anandamide-like cannabinoids in other in vivo bioassays, including the tetrad tests, cannabinoid discrimination, and self-administration, suggesting the likelihood of fundamental differences between at least some of the mechanisms underlying the pharmacology of anandamide versus that of classical cannabinoids such as Δ^9-THC.

2 In Vitro Models

Until the discovery and cloning of CB1 cannabinoid receptors in the brain and CB2 cannabinoid receptors in the periphery, early studies of cannabinoids relied almost exclusively upon in vivo methods to characterize cannabinoids. We now know that these initial animal models, as described in the preceding section, were identifying agents that acted at the CB1 cannabinoid receptor. The later identification of an endocannabinoid system further expanded the scope of in vitro, in situ, and ex vivo models for identifying agents that act at different sites within this system. The major focus of these in vitro bioassays for cannabinoids has been on characterization of the receptor-binding properties of agonists and antagonists for CB1 and CB2 cannabinoid receptor subtypes. These binding assays, when combined with appropriate functional assays, allow for distinguishing full and partial agonists from antagonists and inverse agonists.

2.1 CB1 Cannabinoid Receptor Binding

The most predominant early hypothesis for the mechanism through which Δ^9-THC and other psychoactive cannabinoids produced their diverse profile of in vivo effects was disturbance of the composition of neuronal cell membranes. Through this *perturbation of cell membranes*, it was believed that Δ^9-THC interfered with the normal action of many neurotransmitters relatively nondiscriminantly. The very high lipophilicity of cannabinoids was cited as support for this hypothesis. In addition, early efforts to develop a receptor-binding assay for cannabinoids were minimally successful. In one of the first such studies, Harris et al. [101] used radiolabeled Δ^8-THC in an effort to characterize a binding site in brain and liver, but obtained specific binding in

the latter tissue only. Similarly, Nye et al. [102] described a binding site in the brain with the use of a trimethylammonium derivative, but many psychoactive cannabinoids failed to bind to this site. Lack of specificity and selectivity in these binding assays did nothing to refute the *membrane perturbation* theory of cannabinoid action.

Nevertheless, increasing evidence from in vivo studies suggested that the effects of Δ^9-THC in the brain were *not* so indiscriminant. Irrefutable evidence that Δ^9-THC's psychoactivity was receptor mediated was provided when Allyn Howlett's laboratory successfully added a radiolabel to a potent synthetic cannabinoid, CP 55,940, and reported favorable incubation conditions for a binding assay with this radiolabeled ligand [103]. Radiolabeling involves addition of a radioactive ion (e.g., ^{3}H) to a compound. Once labeled, the presence of the compound can be detected through measurement of radioactivity. Initial binding studies with [^{3}H]CP55,940 demonstrated that this high-affinity ligand binds to *specific* receptors offering support for the existence of cannabinoid receptors in the brain. We now know that [^{3}H]-CP 55,940 binding in brain is predominately, if not exclusively, to the CB1 receptor. Howlett et al. [104] found an excellent correlation between inhibition of adenylyl cyclase and competition for [^{3}H]-CP 55,940 binding in brain tissue for cannabinoid stereoisomers, suggesting stereospecificity of agonists at the binding site. Subsequently, several laboratories [105, 106] established an excellent correlation between [^{3}H]-CP 55,940 binding in rat brain and in vivo pharmacological potency for a series of tetrahydrocannabinol and bicyclic compounds, which had the dual effect of establishing the pharmacological relevance of this binding site for Δ^9-THC and other psychoactive derivatives of the marijuana plant and of verifying the cannabinoid nature of the bicyclic compounds.

This binding assay has also been critical in identifying new classes of compounds as cannabinoids; for example, aminoalkylindoles and anandamides. The discovery that aminoalkylindoles exhibited cannabinoid properties in vitro [107, 108] and in vivo [32] led to the use of [^{3}H]-WIN 55,212-2 as a new ligand for receptor binding [109]. Moreover, the [^{3}H]-CP 55940 assay was critical in identifying the endogenous ligands anandamide [110] and 2-arachidonoylglycerol [111]. Although WIN 55,212-2 does not bind to the CB1 receptor in a fashion identical to that of traditional cannabinoids [112], major differences in the competition of CB1 cannabinoid receptor agonists and antagonists for displacement of [^{3}H]-CP 55,940 versus [^{3}H]-WIN 55,212-2 binding have not been reported. However, it should be pointed out that several reports suggest that WIN 55212-2 might interact with a brain receptor other than the CB1 receptor [113, 114].

In addition to its role in the identification and characterization of cannabinoid agonists, the [^{3}H]-CP 55,940 binding assay was also instrumental in the development of the first selective antagonist of the CB1 receptor. Using [^{3}H]-CP 55,940 binding as a screening tool, SR 141716 was found to bind with high affinity to CB1 receptors in rat brain. Coupling this finding with in vitro functional assays and blockade of cannabinoid effects in vivo established that

it was an antagonist [10]. This antagonist was then radiolabeled which provided a means for characterizing CB1 receptor binding using both agonists and antagonists [115]. As expected for antagonists, [^{3}H]-SR 141716 binding was insensitive to temperature changes and the presence of nonhydrolyzable guanylnucleotide while agonist ([^{3}H]-CP 55,940) binding is sensitive to both conditions. In addition, agonists such as CP 55,940, WIN 55,212-2, and Δ^9-THC exhibited lower affinity for [^{3}H]-SR 141716 binding than for [^{3}H]-CP 55,940 binding. On the other hand, SR 141716 had higher affinity for [^{3}H]-SR 141716 binding. Despite this very limited series of compounds, it appeared that rank order potencies differed between the two assays. These findings were extended by Thomas et al. [116] who conducted a direct comparison between [^{3}H]-SR 141716 and [^{3}H]-CP 55,940. These researchers reported that rank order potencies of the compounds tested for displacement of [^{3}H]-CP-55,940 binding corresponded with those from previous [^{3}H]-CP-55,940 studies. However, the rank order potencies of these compounds in competition studies with [^{3}H]-SR141716 differed significantly from those determined with [^{3}H]-CP-55,940. While these studies demonstrate that CP 55,940 and SR 141716 do not interact with the CB1 receptor in an identical fashion, both ligands are capable of distinguishing between cannabinoid and noncannabinoid analogs.

Since rat brain represents an excellent source of CB1 cannabinoid receptors at a very reasonable cost, it has been an attractive option for conducting large-scale binding, such as that required for structure–activity relationship studies. The drawback to native tissue, however, is the possibility of contamination with other binding sites or unidentified receptors, as mentioned above. In addition, if important differences exist between rat and human CB1 cannabinoid receptors, rat brain tissue might result in results that are misleading in drug development studies. For these reasons, cell lines expressing the human CB1 receptor were constructed [117] in order to obviate these concerns. While we have not conducted an exhaustive investigation comparing binding to rat and human CB1 receptors, we have not observed significant differences between the two. We recently established the receptor affinities for a large series of water-soluble analogs using human CB1 receptors expressed in HEK 293 cells.

The binding assays described above are well suited to establishing the receptor affinity of compounds. However, it is often necessary characterize the location and status of receptors in native tissue, particularly brain. For this type of autoradiographic study, the procedure is essentially the same as that for brain homogenates with the exception that brain slices are incubated with the radiolabeled drug and then juxtaposed to film for development. This technique has been used to map the CB1 receptor in rodent [118] and human brain [119]. It has also been used to localize CB1 receptors pre- and postsynaptically [120]. These studies proved crucial in establishing the relevance of the CB1 cannabinoid receptor to specific cannabinoid actions and the role of the endocannabinoid system in brain function.

In addition to the acute studies described above, there have been numerous studies documenting CB1 receptor downregulation following chronic exposure

to cannabinoid agonists [88, 121], as well as after repeated exposure to other drugs, such as clozapine [122]. Unfortunately, due to its high lipophilicity, Δ^9-THC is highly prone to tissue sequestration, which can present a confounding factor in receptor binding studies after repeated dosing. Residual levels of cannabinoid in the tissue of tolerant animals may be indicated by an alteration in the receptor affinity of the radiolabeled cannabinoid ligand. Therefore, care must be exerted to ensure that tissue levels of Δ^9-THC have dissipated before conducting the binding analysis. Of course, animals treated with drugs that do not compete with binding to the cannabinoid receptor do not pose a problem.

2.2 CB2 Cannabinoid Receptor Binding

Since the cloning of the CB2 receptor [123], a great deal of effort has gone into characterizing structural requirements for ligands that bind to and activate this receptor. Fortunately in this regard, [^{3}H]-CP 55,940, the primary radiolabeled ligand for CB1 receptor binding assays, also binds with excellent affinity to the CB2 receptor [123]. Indeed, the fact that traditional cannabinoids, such as Δ^9-THC, anandamide, CP 55,940, and WIN 55,212-2, also bind to CB2 receptors played an important role in verifying that the CB2 receptor was a cannabinoid receptor subtype. Therefore, ligands that have been used for CB1 receptor binding have also been used for CB2 receptor binding in native as well as cell culture expression systems. For example, Munro and colleagues measured competitive binding with both [^{3}H]-WIN 55,212-2 and [^{3}H]-CP 55,940 in human CB2 receptor expressed in CHO cells and found that binding was sodium and GTP dependent [123]. Several other laboratories, including our own, have sometimes relied on spleen tissue as a convenient source of CB2 receptors [21, 124]. However, the low abundance of CB2 receptors in spleen (as compared to the abundance of CB1 receptors in brain) and uncertainty as to the presence of other cannabinoid receptors in this tissue make it much less favorable than cell expression systems. Subsequent evaluation of a large series of analogs in both CB1 and CB2 receptor bindings revealed modest CB1 selectivity for anandamide analogs and modest CB2 selectivity for some indole analogs [125]. Since the discovery of CB1 and CB2 subtypes of cannabinoid receptors, there have been numerous studies characterizing the structural requirements that distinguish CB1 and CB2 receptor binding [126–132]. These efforts have resulted in several unique structures that exhibit CB2 selectivity and clinical potential for pain [130, 133, 134].

2.3 Non-CB1/CB2 Binding Assay

There are several lines of evidence suggesting the existence of additional cannabinoid receptor subtypes. Kunos' laboratory has described an

anandamide-sensitive site in the mesentery that is blocked by agents that do not interact with either CB1 or CB2 receptors [135]. A distinctively different site has been proposed in brain with the finding that anandamide and WIN 55,212-2 will stimulate G proteins in the absence of CB1 and CB2 receptors [113]. Recent findings have suggested several orphan G-protein-coupled receptors could be cannabinoid subtypes. The most notable, GPR55, is expressed abundantly in brain [136], and some cannabinoids bind to it [137]. However, considerable challenges must be overcome before it can be ascertained that GPR55 is indeed a cannabinoid receptor with physiological relevance [138].

2.4 TRPV$_1$Receptor Binding

In addition to their effects at cannabinoid receptors, endogenous cannabinoids also interact with TRPV$_1$ receptors. Ross et al. [139] assessed TRPV$_1$ receptor binding directly through displacement of the radiolabeled TRPV$_1$ agonist, [^{3}H]-resiniferatoxin, in CHO cells expressing TRPV$_1$ receptors. This assay allowed them to define some of the structural requirements of cannabinoids for this site. Whereas the identified cannabinoid receptors are of the G-protein-coupled type, TRPV$_1$ receptors are associated directly with cation channels, allowing for indirect methods of determining agonist binding at this site. For example, several laboratories have measured the actions of cannabinoid analogs by measuring calcium influx in HEK 293 cells expressing human TRPV$_1$ receptors [21, 140, 141]. Because calcium influx is contingent upon activation of the receptor, this assay has also been used in the evaluation of functional potency and efficacy at TRPV$_1$ receptors.

2.5 In Vitro and In Situ Assays of G-Protein-Coupled Receptor Function

Binding assays provide a means for determining the affinity of ligands for receptors but reveals little about their pharmacological potency or efficacy. While a ligand must bind to a recognition site on the receptor, it does not have to activate the receptor. Therefore, the above binding assays are ideal for detecting all agents that act at the site that is bound by the radiolabeled ligand regardless of whether they are full, partial, or inverse agonists or antagonists. As it turns out, an excellent structure–activity relationship is often used successfully to relate receptor affinity to pharmacological potency in a defined series of compounds as was the case with bicyclic cannabinoids developed by Pfizer [105]. On the other hand, structural modifications of the CB1 cannabinoid receptor antagonist SR 141716 transform it into agonists that bind to the CB1 receptor as well [11]. These simple illustrations point to the need for

having both binding and functional assays in order to fully characterize ligand–receptor interaction.

Both CB1 and CB2 cannabinoid receptors belong to the class of G-protein-coupled receptors. As such, activation of these receptors results in separation of α and $\beta\gamma$ subunits of the G protein which subsequently interact with effector unit(s) to produce a cascade of intracellular-signaling events. This process is dependent upon binding of the α subunit and GTP and occurs only upon activation of the receptor. Binding of a neutral antagonist to the receptor does not engage this process. Under normal conditions, receptor action is terminated when GTPase activity converts GTP to GDP and the α and $\beta\gamma$ subunits are reassociated; however, substitution of radiolabeled GTP, as in the [^{35}S]-GTPγS assay, interferes with this conversion. This assay has proven to be a convenient tool for measuring activation of CB1 and CB2 receptors, since a cannabinoid must activate the receptor before GTPγS will bind. Once bound to the α subunit, [^{35}S]-GTPγS remains bound due to its resistance to GTPase activity. Therefore, measuring binding of [^{35}S]-GTPγS in the presence of a ligand will reveal the potency and efficacy of that agent to activate the receptor. Selley et al. [142] demonstrated that this technique could be used with cannabinoid receptors in rat brain membranes to identify cannabinoid agonists. [^{35}S]-GTPγS binding to mouse brain membranes was also used to verify the antagonistic properties of AM 630 [143] and, in combination with [^{3}H]-CP 55,940 receptor binding, it has been used to characterize antagonists that bind irreversibly to CB1 cannabinoid receptors [144]. A similar [^{35}S]-GTPγS binding assay has also been used to characterize potency and efficacy of CB2 receptor agonists [132]. This assay is amenable to evaluating large series of compounds of both high and low affinity, as long as the concentrations of GDP are closely regulated. Because this process is studied in isolation, it should be borne in mind that these systems might not provide a true reflection of what is occurring at the cellular level in native tissue.

The [^{35}S]-GTPγS binding assay represents only one of the methods of evaluating cannabinoid receptor function. Measurement of intracellular events that occur downstream of G-protein activation, at the level of the effector unit(s), provides an alternative way to assess functional activity of the receptor. For example, some laboratories prefer to measure adenylyl cyclase activity or cyclic AMP production rather than [^{35}S]-GTPγS binding. Since the CB1 and CB2 receptors inhibit cyclic AMP production, use of this alternative procedure requires artificial stimulation of adenylyl cyclase with an agent such as forskolin followed by measurement of inhibition of this response to determine agonist potency and efficacy [145, 146]. Often, a combination of these functional assays has been used. For example, Rinaldi-Carmona et al. [146] relied on [^{3}H]-CP 55,940 binding and antagonism of CP 55,940 inhibition of forskolin-stimulated adenylyl cyclase in cells expressing the human CB2 cannabinoid receptor to characterize SR 144528 as a CB2 selective antagonist. Similarly, delineation between partial agonists, full agonists, antagonists, and inverse agonists at CB1 and CB2 receptors was achieved by a combination of [^{3}H]-CP 55,940 binding

assays, inhibition or enhancement of forskolin-stimulated cyclic AMP production, and alteration in [^{35}S]-GTPγS binding [147].

Steve Childers' laboratory extended the utility of the [^{35}S]-GTPγS binding assay by developing methodology for conducting concurrent autoradiography, resulting in a report detailing differences in CB1 receptor–G-protein coupling throughout brain [148]. These findings underscore the importance of examining receptor function in brain areas relevant to the specific question that is being posed. These functional assays, particularly when several are used in combination, have considerable power. For example, [^{35}S]-GTPγS binding and autoradiography have been used to evaluate CB1 receptor function throughout brain in rodents exposed repetitively to Δ^9-THC [149, 150], morphine [151], and ethanol [152]. One important finding of these investigations was that Δ^9-THC tolerance resulted in heterologous desensitization of GABA and adenosine regulation of adenylyl cyclase activity without alteration of G-protein function.

A final method that has been used to assess cannabinoid receptor function and deserves mention here is measurement of the effects of cannabinoids on ion channels associated with CB1 and/or CB2 receptor activation. In addition to their other receptor-related actions delineated above, CB1 receptor agonists inhibit N-type calcium channels in NG108-15 cells [153, 154]. They also activate inwardly rectifying potassium channels coexpressed with CB1 receptors in xenopus oocytes [154], which has been shown to be an effective model for determining cannabinoid affinity and efficacy [155].

2.6 Endocannabinoid Metabolism

Although receptor activation and inhibition have been the primary means of manipulating the endocannabinoid system, the identification and cloning of the primary metabolic enzyme for anandamide, fatty acid amidohydrolase (FAAH) [14], has directed attention to alteration of the synthesis and metabolism of endocannabinoids (e.g., anandamide and 2-arachidonoylglycerol) as a means of probing the endocannabinoid system and developing therapeutic agents. Several different approaches have been used to examine FAAH substrate specificity and to develop selective inhibitors. All of these approaches have involved measurement of the rate or degree of FAAH-induced hydrolysis of endocannabinoids and/or their analogs. For example, Boger et al. [156] established FAAH substrate specificity through incubation of a large series of structural analogs of endocannabinoids with recombinant FAAH. Subsequently, they determined the rate of hydrolysis for these analogs by using gas chromatography to quantify substrate and product. Others have relied on measuring the rate of hydrolysis of radiolabeled anandamide by native tissue followed by separation of radioactive substrate and product through solvent extraction [157, 158]. With this method, [^{14}C]-anandamide is rapidly hydrolyzed

by rat brain membranes to yield [^{14}C]-ethanolamine [159]. After the intact of [^{14}C]-anandamide is removed by solvent extraction, the remaining [^{14}C]-ethanolamine in the aqueous phase can be measured and the degree of hydrolysis (or FAAH activity) calculated. This technique has proven to be particularly effective in identifying selective FAAH inhibitors.

The metabolism of 2-arachidonoylglycerol is more complicated, in that it can be hydrolyzed by FAAH [160, 161] and by a monoacylglycerol lipase (MAGL) [161]; however, the latter is probably more important for its metabolism in brain. Despite differences in the metabolic rates of 2-arachidonoylglycerol by these two enzymes, it remains challenging to ensure that any metabolic activity is due solely to one of the enzymes. Taking advantage of the fact that MAGL is both cytosolic and membrane bound whereas FAAH is only membrane bound, Chris Fowler's laboratory measured the hydrolysis of 2 arachidonoylglycerol or oleoylglycerol in the cytosol prepared from cerebella of rats [162]. The fact that multiple forms of MAGL exist must be resolved before specific inhibitors and activators can be developed.

Manipulation of the enzymes responsible for endocannabinoid synthesis is also problematic at this time. Although it was thought that the rate-limiting step in the synthesis of anandamide resides in N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) [163], a recent paper from Ben Cravatt's group [164] revealed that knocking out this enzyme did not alter anandamide levels. They later identified two other putative metabolic pathways [165]. At present, it is premature to describe a method for altering anandamide synthesis given the uncertainty as to which enzymes are critical for its formation.

In contrast, the cloning of diacylglycerol lipases (DAGL) responsible for the synthesis and release of 2-arachidonoylglycerol proved to be a major advance [166]. Membranes from COS cells overexpressing recombinant human DAGLα were used to screen cannabinoid analogs as DAGLα inhibitors, while cytosolic fractions from wild-type COS cells were used to search for MAGL inhibitors. DAGLα and MAGL activities were assessed by using sn-1-[^{14}C]-oleoyl-2-arachidonoylglycerol and 2-[^{3}H]-arachidonoylglycerol as substrates, respectively.

2.7 Anandamide Transporter

Transport of anandamide across synaptic membranes has been extensively discussed. While there is ample evidence to both support and challenge the concept of a specific transporter, the fact that none has been cloned leaves the issue unresolved. The development of AM404 as an anandamide transport inhibitor has attracted most attention following evidence that it inhibited high-affinity anandamide accumulation in rat neurons and astrocytes [167]. It is unfortunate that it also activates vanilloid receptors [168]. However, two types of procedures are relatively straightforward transporter assays. In the first type of procedure, the degree to which the purported transporter

inhibitor alters uptake of [^{3}H]-anandamide in incubation in U937 cells is measured after filtration [169]. In the second type of procedure, disappearance of [^{3}H]-anandamide from the incubation medium containing astrocytoma cells has been used as a measure of transporter activity [170].

3 Conclusion

The discovery of cannabinoid receptors, signaling pathways, endocannabinoid ligands, and endocannabinoid metabolic and degradative enzymes could not have been possible without appropriate methodology for systematically evaluating their properties. Despite the recent dramatic increase in our knowledge concerning cannabinoid receptor binding and function, it is unlikely that all sites have been presently characterized. Undoubtedly, there are additional receptor subtypes, endocannabinoids, and enzymes for the synthesis and metabolism of new endocannabinoids. Strategies used in developing the current cannabinoid in vitro methods will be useful in developing methods for these new targets. Furthermore, the development of procedures for the rapid evaluation of analogs has allowed for the development of highly potent and selective probes for many of the targets in the endocannabinoid system. These compounds hold promise as important therapeutic agents for a wide range of maladies, as demonstrated by concurrent in vivo studies.

Acknowledgments Preparation of this manuscript was supported by National Institute on Drug Abuse grants DA-09789 and DA-03672.

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Therapeutic Applications for Agents that Act at CB1 and CB2 Receptors

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Abstract This chapter reviews evidence that supports the established therapeutic uses of cannabinoid receptor ligands: CB1 and/or CB2 receptor agonists as anti-emetics, appetite stimulants, and analgesics for the relief of neuropathic pain, and CB1 receptor antagonists for the treatment of obesity and related metabolic risk factors. It also identifies and discusses some additional *potential* therapeutic applications of cannabinoid receptor ligands for which the preclinical and clinical evidence is particularly promising. These include the use of cannabinoid receptor agonists for the management of multiple sclerosis and inflammatory pain. In addition, brief mention is made of a range of other possible therapeutic uses for cannabinoid receptor agonists, antagonists, and inverse agonists.

Keywords Δ^9-Tetrahydrocannabinol · Dronabinol · Marinol® · Sativex® · Nabilone · Cesamet® · SR141716A · Rimonabant · Acomplia® · Pain · Multiple sclerosis · Spasticity · Appetite · Nausea · Vomiting · Obesity

1 Introduction

There are at least two types of cannabinoid receptor, both G-protein coupled [1]. These are CB1 receptors that are expressed predominantly at nerve terminals and mediate inhibition of neurotransmitter release, and CB2 receptors that are found mainly on immune cells and modulate cell migration and cytokine release. CB2 receptors have also been detected on central neurons (Sect. 2.1). However, the physiological role of these neuronal CB2 receptors is yet to be established. Endogenous agonists for cannabinoid receptors also exist, the most investigated of these *endocannabinoids* being arachidonoylethanolamide (anandamide) and 2-arachidonoyl glycerol. These are synthesized on

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P.H. Reggio (ed.), *The Cannabinoid Receptors*, The Receptors,
DOI 10.1007/978-1-59745-503-9_13,

demand and removed from their sites of action by cellular uptake. They are then degraded by enzymes that include fatty acid amide hydrolase, monoacyl glycerol lipase (for 2-arachidonoyl glycerol), cyclooxygenase-2, lipoxygenases, and cytochrome P450 [2, 3]. Endocannabinoids and the cannabinoid CB1 and CB2 receptors together constitute the *endocannabinoid system*.

The discovery of cannabinoid receptors was followed by the development of CB1- and CB2-selective agonists and antagonists [1, 4]. These include the CB1-selective agonists, ACEA, ACPA, and methanandamide, and the CB2-selective agonists, JWH-133, JWH-015, HU308, and AM1241 (Table 1). As to CB1- and CB2-selective antagonists, the best known of these are the CB1-selective SR141716A, AM251, AM281, and the CB2-selective SR144528 and AM630 (Table 1). These antagonists all behave as inverse agonists, one indication that CB1 and CB2 receptors can exist in a constitutively active state [5]. CB1- and CB2-selective agonists were developed from compounds that form part of an earlier generation of cannabinoid receptor agonists, the members of which interact more or less equally well with CB1 and CB2 receptors. Prominent examples of such CB1/CB2 agonists are the main psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol (Δ^9-THC), the synthetic compounds, nabilone, 11-hydroxy-Δ^8-THC-dimethylheptyl (HU-210), CP55940, and *R*-(+)-WIN55212, and the endocannabinoids, anandamide, and 2-arachidonoyl glycerol (Table 1). All the above cannabinoid receptor agonists and antagonists have been used in experiments described in this chapter, as have the recently developed CB1-selective antagonist/inverse agonist, SR147778 [6], and CB2-selective agonist, GW405833 [7] (Table 1). As well as activating or blocking the CB1 receptor with ligands that bind to its *orthosteric site* it should soon also be possible to enhance or attenuate CB1 receptor activation less directly by targeting an allosteric site recently discovered to be present on this receptor [8].

Table 1 Some cannabinoid receptor ligands

CB1-selective agonists	CB2-selective agonists	CB1/CB2 agonists
ACEA	JWH-133	Δ^9-THC
ACPA	JWH-015	Nabilone
Methanandamide	HU-308	HU-210
	AM1241	CP55940
	GW405833	*R*-(+)-WIN55212
		Anandamide
		2-Arachidonoyl glycerol
CB1-selective antagonists/inverse agonists	CB2-selective antagonists/inverse agonists	
SR141716A	SR144528	
AM251	AM630	
AM281		
SR147778		

The structures of these ligands and descriptions of their pharmacological properties can be found elsewhere [1, 4, 6, 7].

There is convincing evidence that some CB1/CB2 receptor agonists also have non-CB1 and non-CB2 targets, which for anandamide include the vanilloid TRPV1 receptor [4]. Not all cannabinoid receptor agonists appear to interact with each of these putative additional targets to the same extent. Consequently, they are likely to possess different pharmacological profiles in spite of their shared ability to activate CB1 and/or CB2 receptors, something that should be borne in mind when selecting a cannabinoid receptor agonist for use as a pharmacological tool or potential medicine.

This chapter focuses in part on *actual* therapeutic uses of cannabinoid receptor agonists and antagonists and the evidence that supports these uses (Table 2). Some of this evidence relates to data obtained with two CB1/CB2 receptor agonists that have been licensed as medicines since the 1980 s. These are Δ^9-THC (dronabinol; Marinol®) and nabilone (Cesamet®), a synthetic analogue of Δ^9-THC, both of which are administered orally in capsules. Δ^9-THC and nabilone are prescribed for the suppression of nausea and vomiting provoked by cancer chemotherapy and Δ^9-THC is also prescribed as an appetite stimulant particularly to AIDS patients who are experiencing excessive loss of body weight. Also described are data obtained with the cannabis-based medicine, Sativex®, and with the CB1-selective antagonist/inverse agonist, SR141716A. Sativex® was licensed in Canada in 2005 as adjunctive treatment for the symptomatic relief of neuropathic pain in adults with multiple sclerosis and is delivered to patients by a pump action oromucosal spray. The main constituents of Sativex® are Δ^9-THC and the nonpsychoactive plant cannabinoid, cannabidiol [9]. SR141716A (rimonabant; Acomplia®) was licensed in the UK in 2006 for the treatment of obesity and related metabolic risk factors.

There is also some discussion in this chapter of *potential* clinical applications of cannabinoid receptor ligands for which the preclinical and clinical evidence is particularly promising. These include the use of cannabinoid receptor agonists for the management of multiple sclerosis and of inflammatory pain (Table 2). In addition to the therapeutic applications listed in Table 2, there are a number of other therapeutic uses that have been proposed for cannabinoid receptor

Table 2 Proven and promising therapeutic targets for CB1 and/or CB2 receptor agonists and antagonists

Targets for CB1 and/or CB2 receptor agonists	Section
Postoperative pain or chronic neuropathic or inflammatory pain experienced by patients with disorders such as multiple sclerosis, spinal cord injury, brachial plexus injury, or cancer	2.1
Spasticity, spasms, and certain other symptoms experienced by patients with multiple sclerosis or spinal cord injury	2.2
Nausea and vomiting induced by cancer chemotherapy	2.3
Loss of appetite in patients with cancer or AIDS	2.4
Targets for CB1 receptor antagonists	
Obesity	2.5

Table 3 Some potential therapeutic targets for CB1 and/or CB2 receptor agonists

Targets	Reference
Disturbed behavior/agitation in patients with Alzheimer's disease	[10]
Anxiety disorders	[10]
Attention-deficit hyperactivity disorder (ADHD)	[11, 12]
Tics and behavioral problems in patients with Tourette's syndrome	[10, 13]
Tardive dyskinesia induced by neuroleptic drugs in psychiatric patients	[13]
Amyotrophic lateral sclerosis	[14, 15]
Gastrointestinal disorders, including gastric ulcers, cholera-induced diarrhea, inflammatory bowel disease and gastro-esophageal reflux disease	[16–18]
Inhibition of angiogenesis and growth of tumors including gliomas and lymphomas and lung, breast, colorectal, thyroid, prostate and skin cancer cells	[16, 19]
Hypertension	[3]
Hemorrhagic and cardiogenic shock	[3]
Atherosclerosis	[3
Glaucoma	[10, 20]
Cough	[21, 22]
Cholestatic pruritus	[23, 24]

ligands (Tables 3–5). For two of these, the treatment of Parkinson's disease and the management of dyskinesia induced by L-dopa in patients with this disease, it is currently unclear whether it would be best to administer a cannabinoid receptor agonist or antagonist (Table 5). The possibility also exists that there are some disorders, for example, inflammation and osteoporosis, for which either a cannabinoid receptor agonist or a cannabinoid receptor antagonist/ inverse agonist would serve as an effective treatment (Table 5). The potential therapeutic applications listed in Tables 3–5 are not discussed further in this chapter.

Table 4 Some potential therapeutic targets for CB1 antagonists/inverse agonists

Target	Reference
Drug dependence, particularly nicotine dependence	[25]
Impaired fertility in some women	[3]
Stroke	[3]
Hypotension of endotoxemic shock triggered by advanced liver cirrhosis	[3]
Intestinal hypomotility in paralytic ileus	[3, 16]

Table 5 Disorders or symptoms that might be ameliorated either by a CB1 or CB2 receptor agonist or by a CB1 or CB2 receptor antagonist/inverse agonist

Disorder or symptom	Reference
Motor impairment and tremor in Parkinson's disease	[10, 13]
Dyskinesia induced by L-dopa in patients with Parkinson's disease	[13]
Inflammation	[3, 26, 27]
Osteoporosis	[3, 28, 29]

2 Proven and Promising Therapeutic Applications for Cannabinoid Receptor Agonists and Antagonists

2.1 Pain

It is well established that activation of cannabinoid CB1 receptors located on neurons in the brain and spinal cord as well as on peripheral sensory neurons can induce antinociception in a wide range of preclinical models of pain. Indeed, as has been discussed in greater detail elsewhere [30–34], it is now generally accepted first that CB1 receptors are present on pain pathways in the brain and spinal cord and on the central and peripheral terminals of primary afferent neurons that mediate pain and second that CB1/CB2 receptor agonists such as Δ^9-THC, HU-210, *R*-(+)-WIN55212 and anandamide can induce signs of antinociception in mouse, rat, or monkey models of acute thermal pain, inflammatory pain, and neuropathic pain. This they can do either when administered peripherally or when injected directly onto a CB1 receptor-expressing region of a pain pathway located in the brain or spinal cord or outside the central nervous system, for example, in the paw of a rodent or the tail of a monkey. It is also well established that the antinociceptive activity of these agonists can be counteracted by the CB1-selective antagonist, SR141716A, albeit sometimes in pain models in which this compound produces signs of hyperalgesia when administered by itself, an effect that could reflect either antagonism of endocannabinoids released onto CB1 receptors or SR141716A-induced inverse agonism, or both of these actions.

That CB1 receptors have a role in pain perception is also supported by reports first that the CB1-selective agonist, ACEA, induces electrophysiological signs of antinociception in rat spinal neurons in a model of inflammatory pain [35], second that mice from which the CB1 receptor has been genetically deleted ($CB1^{-/-}$ mice) exhibit tactile hypersensitivity [36], and third that mice in which antisense methods have been used to achieve a *knockdown* of CB1 receptors in the brain or spinal cord exhibit signs of hyperalgesia and reduced sensitivity to CB1/CB2 receptor agonist-induced antinociception in models of acute thermal pain [37–39]. There are also reports, however, that when compared to wild-type animals, $CB1^{-/-}$ mice exhibit no differences in thermal paw-withdrawal latency [36] or in nociceptive behavior in hot plate, tail-immersion, tail flick, tail pressure, or abdominal stretch tests, or even show signs of hypoalgesia in hot plate and formalin paw tests [40].

There is evidence from experiments with either CB2-selective agonists (AM1241, HU308, GW405833, and JWH-133) or a CB1/CB2 receptor agonist (CP55940) that CB2 receptors can also mediate analgesia. More specifically, there are reports that antinociceptive responses can be elicited

1. by AM1241 in rat models of inflammatory and acute thermal pain and in mouse and rat models of neuropathic pain in a manner that can be opposed by the CB2-selective antagonists, SR144528 or AM630, but not by the CB1 selective antagonists, SR141716A or AM251 [36, 41–44];

2. by HU308 in the second phase of the mouse formalin paw test in a manner that is sensitive to antagonism by SR144528 but not SR141716A [45];
3. by GW405833 in rat and mouse models of chronic inflammatory pain and of neuropathic pain [7, 46];
4. by GW405833 and JWH-133 in the rat carrageenan model of inflammatory pain in a manner that can be blocked by SR144528 (both compounds) but not SR141716A (JWH-133) [47, 48];
5. by AM1241, GW405833, and HU308 in a rat hindpaw incision model of postoperative pain in a manner that, for HU308, can be blocked by SR144528 [7, 49];
6. by CP55940 in a manner that is sensitive to antagonism by SR144528 but not SR141716A in a rat model of neuropathic pain and to antagonism by both SR141716A and SR144528 in a rat model of acute thermal pain [50].

It is also noteworthy first that Ibrahim et al. [36] have demonstrated AM1241 to be no less effective in reducing signs of neuropathic pain in $CB1^{-/-}$ mice than in wild-type animals and second that GW405833 has been found to reduce signs of chronic inflammatory pain in wild-type mice but not in $CB2^{-/-}$ animals [7, 46].

Results obtained in experiments with a rat model of acute thermal pain have prompted the hypothesis that AM1241 may produce antinociception by acting on CB2 receptors expressed by skin keratinocytes to trigger the release of β-endorphin from these non-neuronal cells onto μ-opioid receptors located on the terminals of primary afferent neurons [51]. Against this hypothesis, however, is evidence obtained recently by Whiteside et al. [46] that the CB2-selective agonist, GW405833, induces antinociception in rat models of acute thermal pain and chronic inflammatory pain that is not dependent on the release of endogenous opioids. It is also possible that CB2 receptor agonists can induce antinociception by activating CB2 receptors expressed by microglial cells or even by neurons. Thus, there is evidence first that after peripheral nerve injury, CB2 receptors appear in rat paw skin and on neurons and activated microglia in mouse or rat spinal cord [52–54] and second that CB2 receptors are expressed both by primary cultures of neonatal rat dorsal root ganglion neurons [55] and by brain stem neurons of unlesioned rats, ferrets, and mice, cerebellar neurons of unlesioned rats and ferrets and cerebral cortical neurons of unlesioned rats [56, 57]. CB2-selective agonists may also produce antinociception by targeting *CB2-like* receptors, putative receptors that may mediate hypoalgesia induced by the endogenous fatty acid amide, palmitoylethanolamide [4, 31].

Turning now to evidence that cannabinoid receptor activation can induce antinociception in human subjects, the first tranche of clinical data came from trials conducted between 1975 and 1996, usually with rather small numbers of patients [58, 59]. These early investigations yielded data suggesting that an intramuscular injection of the cannabinoid receptor agonist, L-nantradol, can relieve postoperative pain [60]; orally administered Δ^9-THC can relieve cancer pain when administered at doses of 10, 15, or 20 mg [61, 62] and painful spasticity caused by spinal cord injury when administered at 5 mg/d [63]; and

that orally administered Δ^9-THC (10 or 15 mg) or nabilone (1 mg alternate day or twice daily) or rectally administered Δ^9-THC-hemisuccinate can relieve pain produced by multiple sclerosis [64–66].

Results from more recent clinical studies are summarized in Table 6. Taken together, the available data convincingly support the argument for using cannabis-based medicinal extracts such as Sativex® (Sect. 1) or cannabinoid receptor agonists such as Δ^9-THC (dronabinol; Marinol®) or nabilone (Cesamet®) as analgesics, particularly for the management of neuropathic pain. The use of cannabinoids for pain relief is also supported by anecdotal evidence, both ancient [83] and more recent, the latter coming particularly from patients with multiple sclerosis, spinal cord injury, sickle cell disease, or pain caused by trauma and/or surgery who choose to self-medicate with cannabis [58, 84–89].

2.2 Multiple Sclerosis

There is convincing preclinical evidence that cannabinoid receptor activation can ameliorate some of the characteristic signs of multiple sclerosis. This comes from experiments with rodent models of multiple sclerosis in which demyelination is induced by inoculation either with Theiler's murine encephalomyelitis virus (TMEV) or with mixtures containing CNS tissue or myelin basic protein that give rise to chronic relapsing experimental allergic encephalomyelitis/experimental autoimmune encephalomyelitis (CREAE/EAE). The main findings obtained from such experiments can be summarized as follows:

1. Repeated administration of Δ^8-THC, Δ^9-THC, or *R*-(+)-WIN55212 to mice, rats, or guinea-pigs with EAE can reduce the clinical severity of this syndrome as measured, for example, by atonia, ataxia, gait abnormalities, hind limb weakness, paraparesis (partial hindlimb paralysis), moribundity, and mortality [90–92];
2. Repeated administration of the CB1/CB2 receptor agonist *R*-(+)-WIN55212, the CB1-selective agonist ACEA and the CB2-selective agonist JWH-015 can improve rotarod performance in TMEV-infected mice [93];
3. *R*-(+)-WIN55212 can reduce spasticity and tremor in CREAE mice after a single injection [94] and the incidence and severity of gait abnormalities, paralysis, and moribundity in TMEV-infected mice [95];
4. The ability of *R*-(+)-WIN55212 to reduce spasticity in CREAE mice is shared by the CB1/CB2 agonists Δ^9-THC, anandamide, and 2-arachidonoyl glycerol, by the CB1-selective agonist methanandamide and by the CB2-selective agonist JWH-133 [94, 96–98], but not by cannabidiol [94, 98], a cannabinoid that lacks significant CB1 or CB2 receptor efficacy or affinity;
5. Spasticity or other signs of impaired motor function can be reduced in CREAE and TMEV-infected mice by *R*-(+)- but not by *S*-(–)-WIN55212 [94, 95] indicating that the ability of *R*-(+)-WIN55212 to ameliorate signs of motor dysfunction in these animals is determined by its molecular conformation;

Table 6 Effects of cannabis, cannabis extracts, or individual cannabinoids on pathological pain: results from clinical trials published between 2000 and 2005

Signs and symptoms/disorder	Design[a]	Drug treatment	Outcome measure and result	Reference
Positive results				
Pain from multiple sclerosis	Randomized, double-blind, placebo-controlled, parallel-group multicentre study (n = 129 to 148 per group)	Marinol® or Cannador p.o. twice daily (maximum daily dose of Δ^9-THC was 25 mg)	Pain rating scale: significant analgesia[b]	[67]
Central neuropathic pain from multiple sclerosis	Randomized, double-blind, placebo-controlled, crossover study (n = 24)	5 mg Marinol® p.o. twice daily	11-Point numerical pain rating scale: significant analgesia	[68]
Pain from multiple sclerosis, spinal cord injury, brachial plexus damage or limb amputation	Randomized, double-blind, placebo-controlled, single-patient crossover study (n = 12)	Sativex®, high-Δ^9-THC, low-CBD cannabis extract or low-Δ^9-THC, high-CBD cannabis extract (oromucosal spray; maximum permitted 24-hourly dose of each cannabinoid was ca. 120 mg)	VAS: no significant efficacy for Sativex®; significant efficacy for the other two cannabis extracts[b]	[69]
Pain from multiple sclerosis in patients with troublesome lower urinary tract symptoms	Open label study (n = 10 or 12)	Sativex® or high-Δ^9-THC, low-CBD cannabis extract (oromucosal spray; maximum permitted daily dose of each cannabinoid was ca. 120 mg)	VAS: significant analgesia (both cannabis extracts)[b]	[70]
Central neuropathic pain from multiple sclerosis	Randomized, double-blind, placebo-controlled, parallel-group study (n = 34 and 32 per group)	Sativex® (oromucosal spray; maximum permitted 24-hourly dose was 129.6 mg for Δ^9-THC and 120 mg for CBD)	11-Point numerical pain rating scale: significant analgesia[b]	[71]

Table 6 (continued)

Signs and symptoms/disorder	Design[a]	Drug treatment	Outcome measure and result	Reference
Chronic pain (mainly neuropathic pain)	Randomized, double-blind, placebo-controlled, crossover trial (34 'N of 1' studies)	Sativex®, high-Δ^9-THC, low-CBD cannabis extract or low-Δ^9-THC, high-CBD cannabis extract (oromucosal spray)	VAS: significant analgesia (Sativex® and high-Δ^9-THC, low-CBD cannabis extract)[b]	[72]
Central neuropathic pain from brachial plexus avulsion	Single centre, double-blind, randomized, placebo-controlled, three period crossover study ($n = 48$)	Sativex® or high-Δ^9-THC, low-CBD-extract (oromucosal spray; maximum permitted 24-hourly dose was 129.6 mg for Δ^9-THC and 120 mg for CBD)	11-Point numerical pain rating scale: significant analgesia (both cannabis extracts)[b]	[73]
Chronic neuropathic pain	Randomized, double-blind, placebo-controlled, crossover study ($n = 21$)	20 mg then 40 mg ajulemic acid p.o. twice daily	(a) VAS: significant analgesia (b) VRS: no significant analgesia (c) mechanical hypersensitivity: no significant efficacy	[74, 75]
Pain from rheumatoid arthritis	Randomized, double-blind, placebo-controlled, parallel-group study ($n = 31$ and 27 per group)	Sativex® (oromucosal spray; mean daily dose was 14.6 mg for Δ^9-THC and 13.5 mg for CBD)	11-Point numerical pain rating scale: significant analgesia; also improved morning stiffness and improved morning pain on movement and at rest[b]	[76]
Severe eye pain with bletharospasm	Open label ($n = 1$)	25 mg Δ^9-THC (dronabinol) p.o. daily	11-Point numerical pain rating scale: significant analgesia	[77]
Acute thermal pain	Placebo controlled ($n = 5$)	Smoked cannabis	Latency of finger withdrawal from radiant heat stimulus: significant analgesia	[78]

Table 6 (continued)

Signs and symptoms/disorder	Design[a]	Drug treatment	Outcome measure and result	Reference
Negative results				
Pain from multiple sclerosis	Randomized, double-blind, placebo-controlled, parallel-group study ($n = 18$ per group)	Sativex® (oromucosal spray; maximum daily dose of Δ^9-THC was ca. 120 mg)	VAS: no significant analgesia[b]	[79]
Chronic refractory neuropathic pain	Open label ($n = 7$)	Marinol® p.o. twice daily (maximum daily dose of Δ^9-THC was 25 mg)	VAS: no significant analgesia[c]	[80]
Refractory central or peripheral neuropathic pain	Open label ($n = 8$)	Marinol® p.o. twice daily (maximum daily dose of Δ^9-THC was 25 mg)	VAS: no significant analgesia[c]	[81]
Postoperative pain from elective abdominal hysterectomy	Randomized, double-blind, placebo-controlled, single-dose parallel-group study ($n = 20$ per group)	5 mg Δ^9-THC p.o.	VAS: no significant analgesia	[82]

CBD cannabidiol, *Δ^9-THC* Δ^9-tetrahydrocannabinol, *VAS* visual analogue scale, *VRS* verbal rating scale. Marinol® is prepared from synthetic Δ^9-THC while Sativex® and Cannador are both cannabis extracts. Ajulemic acid is 1′,1′-dimethylheptyl-Δ^8-THC-11-oic acid

[a] Numbers of patients before any withdrawals

[b] Quality of sleep was improved

[c] Three [80] or five patients [81] withdrew because of adverse effects. There was no unacceptable incidence of adverse effects in the larger clinical trials featured in this table.

6. *R*-(+)-WIN55212-induced amelioration of motor dysfunction in CREAE or EAE mice is attenuated by selective CB1 and/or CB2 receptor antagonists: in CREAE mice amelioration of spasticity by *R*-(+)-WIN55212 is opposed both by SR141716A and by SR144528, albeit at doses of these antagonists that by themselves exacerbate spasticity in these lesioned animals [94, 97], while interestingly in EAE mice *R*-(+)-WIN55212-induced amelioration of tail limpness, hind limb weakness, hind limb paralysis, and moribundity is antagonized by SR144528, but not by SR141716A [91];
7. Compared to wild-type CREAE mice, $CB1^{-/-}$ CREAE mice exhibit an earlier onset of spasticity, more immobility and residual paresis, and greater mortality [99].

Results from animal experiments suggest that as well as ameliorating unwanted signs of multiple sclerosis, cannabinoid receptor activation may oppose the progression of this disease. More specifically, there are reports that administration of *R*-(+)-WIN55212 in vivo can decrease both in vitro secretion of IFN-γ by T lymphocytes taken from TMEV-infected mice and expression of mRNA for several proinflammatory mediators in the spinal cord of TMEV-infected mice [95]. It has also been reported that repeated administration of Δ^9-THC [90], although not of Δ^8-THC [92] can ameliorate spinal cord inflammation in rat or guinea-pig EAE, and that *R*-(+)-WIN55212, the CB1-selective agonist, ACEA, and the CB2-selective agonist, JWH-015, can reduce demyelination, microglial activation, and the number of $CD4^+$ infiltrated T cells in spinal cord tissue of TMEV-infected mice [93]. In addition, it has been found that signs of apoptosis and of axonal damage associated with IFN-γ-induced demyelination are more marked in cultured brain cells from $CB1^{-/-}$ mouse fetuses than from wild-type animals [100] and that $CB1^{-/-}$ CREAE mice exhibit greater mortality and more spinal cord axonal loss, demyelination, and neurodegeneration than wild-type CREAE mice [99, 101]. It has also been discovered that leukocyte rolling and adhesion, two leukocyte–endothelial interactions that are thought to contribute to the progression of EAE (and of multiple sclerosis), can be attenuated in vivo in cerebral venous microvessels of EAE mice by repeatedly administered *R*-(+)-WIN55212 in a manner that is antagonized by SR144528 but not by SR141716A [91]. In summary, the data obtained from experiments with rodent TMEV, EAE, and CREAE models of multiple sclerosis suggest that both the in vivo signs of encephalomyelitis detectable in these models and the progression of encephalomyelitis can be opposed by single or repeated cannabinoid CB1 and CB2 receptor activation. There is, however, still some confusion in the literature as to the relative importance of these two receptor subtypes in the attenuation both of the signs of encephalomyelitis and of its progression.

It may be that any amelioration of TMEV infection, EAE or CREAE mediated by the CB1 receptor results from its ability to attenuate excessive neurotransmitter release when activated and so, for example, protect against excitotoxicity [102]. In TMEV-infected mice at least, some amelioration of the

signs or progression of encephalomyelitis may also result from CB1 receptor-mediated changes in immune function [93], a possibility that is supported by the presence of CB1 receptors in immune cells [103].

As to CB2 receptors, the findings of Arévalo-Martín et al. [93] and Ni et al. [91] described above raise the possibility that activation of CB2 receptors on immune cells may oppose the progression of murine encephalomyelitis. There is also evidence that activation of CB2 receptors can inhibit proinflammatory cytokine release and enhance anti-inflammatory cytokine release from immune cells and trigger chemokinetic and chemotaxic migration of microglial cells [4, 103]. It is noteworthy, therefore, that Maresz et al. [104] have found CB2 but not CB1 mRNA expression to increase markedly in spinal activated microglial cells and peripheral macrophages of mice during EAE onset. It is also noteworthy, however, that neither Δ^9-THC (Marinol®) nor cannabis extract (Cannador) have been found to influence serum cytokine levels in multiple sclerosis patients [105]. Finally, although there is currently no evidence that CB2 receptors share the ability of CB1 receptors to modulate neurotransmitter release, this possibility should not yet be rejected, as CB2 receptors have been detected in mouse and rat spinal neurons following nerve injury [53], in primary cultures of neonatal rat dorsal root ganglion neurons [55] and in brain neurons of unlesioned animals [56, 57] (Sect. 2.1).

The preclinical evidence that cannabinoid receptor agonists have therapeutic potential for the management of multiple sclerosis is supported by an ever-growing amount of clinical data. Briefly, there have been reports that in patients with multiple sclerosis, subjectively perceived spasms and/or spasticity were reduced by

1. 7.5 mg Δ^9-THC p.o. ($n = 8$) in a paired double-blind, placebo-controlled, crossover trial [106];
2. 5 mg Δ^9-THC p.o. ($n = 1$) in a double-blind, placebo-controlled trial [63];
3. 1 mg nabilone p.o. every second day ($n = 1$) in a double-blind, placebo-controlled, crossover trial [65];
4. Δ^9-THC (Marinol®), cannabis extract (Cannador), or placebo p.o. twice daily in a randomized, double-blind, parallel group, multicentre study in which the maximum daily dose of Δ^9-THC was 25 mg (n per group = 167, 180, and 173, respectively for spasms and 176, 184, and 183, respectively, for spasticity) [67];
5. Sativex® or a cannabis extract rich in Δ^9-THC but not in cannabidiol administered as an oromucosal spray in randomized, double-blind, placebo-controlled, single-patient crossover ($n = 14$) [69] or open label studies ($n = 13$) [70] in which the maximum permitted daily dose of Δ^9-THC was 120 mg;
6. Sativex® (oromucosal spray) in a randomized, double-blind, placebo-controlled, parallel group study in which the maximum permitted daily dose of Δ^9-THC was again 120 mg ($n = 80$ per group) [79];
7. Cannabis extract p.o. in a randomized, double-blind, placebo-controlled, crossover study in which the mean daily dose of Δ^9-THC was 7.5 to 27.5 mg ($n = 37$) [107].

Attempts to detect cannabinoid-induced reductions in spasticity in patients with multiple sclerosis objectively have met with limited success, probably because the available objective measures, for example, the Ashworth scoring system are rather insensitive. Negative results have been obtained by Ungerleider et al. [106] with oral Δ^9-THC and by Zajicek et al. [67] with both Marinol® and Cannador at doses that did, however, lessen patient-reported spasticity. Negative results have also been obtained by Vaney et al. [107] with cannabis extract capsules at dose levels that did decrease spasm frequency, and by Killestein et al. [108], albeit with rather low oral doses of Marinol® and cannabis extract that failed even to produce detectable subjective amelioration of spasticity. There are, however, some reports that objectively measured spasticity can be reduced by cannabinoids in multiple sclerosis patients, positive results having been obtained with inhaled cannabis ($n = 1$) in an open label trial [109], with 5 or 10 mg Δ^9-THC p.o. ($n = 9$) in a double-blind, placebo-controlled, crossover trial [110], and with 10 or 15 mg THC p.o. and Δ^9-THC hemisuccinate given rectally ($n = 2$) in an open label trial [64]. In addition, Δ^9-THC, nabilone, cannabis or cannabis extract-induced improvements have been reported for other symptoms of multiple sclerosis. These include pain relief (Sect. 2.1) and amelioration of the following symptoms:

1. quality of sleep [63, 67, 70, 71];
2. ataxia [109];
3. nystagmus and visual acuity [111];
4. mobility in some studies [107, 109] but not in others [67, 79, 108];
5. walk time [64, 67];
6. tremor in some studies [107, 109, 112] but not in others [67, 70, 79, 113];
7. handwriting performance [112]; and
8. urinary function in some studies [63, 65, 70] but not in others [64, 67, 69, 79].

No unacceptable adverse effects of pure cannabinoids, cannabis extracts, or cannabis were detected in any of the clinical trials that have been mentioned in this section.

Anecdotal data obtained by sending out questionnaires to patients lend further support to the conclusion that cannabis ameliorates both signs and symptoms of multiple sclerosis, including spasticity, tremor, and pain [58, 84, 86, 87, 89, 114], and signs and symptoms of spinal cord injury [114–116].

2.3 Nausea and Vomiting

Cancer chemotherapy frequently induces intense and persistent vomiting as well as a marked sensation of nausea. These constitute major adverse drug reactions that often interfere with the successful completion of treatment. In view of this problem, the validity of anecdotal claims that cannabis has antiemetic properties, and indeed, that it is effective against chemotherapy-induced

nausea and vomiting [117, 118] were investigated in the 1970 s and 1980 s in clinical trials, mainly with orally administered Δ^9-THC or nabilone. The resulting data were sufficiently favorable to justify the introduction of Δ^9-THC (dronabinol; Marinol®) and nabilone (Cesamet®) into the clinic in the 1980 s as orally administered antiemetics. Thus, as detailed elsewhere [119, 120], the results obtained in these clinical studies indicated that both these cannabinoid receptor agonists are more effective against nausea and vomiting induced by chemotherapy in cancer patients than dopamine-receptor antagonists such as prochlorperazine. It was also found that patients preferred cannabinoids to dopamine receptor antagonists as antiemetics [120]. Adverse reactions to cannabinoids noted in these clinical studies by some patients included dizziness, hypotension, dysphoria/depression and, less commonly, hallucinations and paranoia. In view of these reported adverse effects in adult patients, it is noteworthy that in one clinical trial, performed with 3–13-year-old cancer patients, it was found that Δ^8-THC-containing oil dropped onto the tongue was highly effective against chemotherapy-induced vomiting but induced no adverse side effects of any note [121].

Although Δ^9-THC and nabilone may have advantages over dopamine receptor antagonists for the management of chemotherapy-induced nausea and vomiting, there have been no reports of any comparisons made in the clinic between either of these cannabinoids and 5-HT_3 receptor antagonists such as ondansetron. However, it has been found that smoked cannabis is less effective than ondansetron, against nausea and vomiting induced in healthy human subjects by syrup of ipecac [122].

Results from experiments with animals have confirmed that cannabinoid receptor agonists can suppress drug-induced vomiting and suggest that these drugs produce this effect by targeting nuclei located in the dorsal vagal complex of the medulla [16, 119, 123]. Animal experiments have also provided evidence that the antiemetic effect of these agonists is mediated by CB1 receptors. Thus, as detailed elsewhere [119], there are reports first that vomiting induced by cisplatin or other emetic agents can be reduced by CB1/CB2 receptor agonists in cats, pigeons, ferrets, least shrews (*Cryptotis parva*), and house musk shrews (*Suncus murinus*), although interestingly not in dogs, and second that reductions in cisplatin-induced retching and/or vomiting produced in ferrets by Δ^9-THC [123] and in the least shrew by Δ^9-THC, CP55940, and *R*-(+)-WIN55212 [124–126] can be attenuated by the CB1-selective antagonist SR141716A. It has also been found that the abilities of cannabinoid receptor agonists (Δ^9-THC, *R*-(+)-WIN55212, anandamide and/or methanandamide) to reduce retching and/or vomiting induced by morphine or morphine-6-glucuronide in ferrets [57, 127, 128], by lithium chloride in the house musk shrew [129] and by 5-hydroxytryptophan in the least shrew [130] are opposed by SR141716A or AM251. It is unlikely that the antiemetic effect of cannabinoids is also CB2 receptor mediated. Thus, it has been reported that reductions of cisplatin-, morphine-, or morphine-6-glucuronide-induced retching and/or vomiting produced by CB1/CB2 receptor agonists in ferrets [57, 123, 127] or least shrews

[124–126] are unaffected by the CB2-selective antagonists SR144528 or AM630. Furthermore, it has been found that morphine-6-glucuronide-induced vomiting in ferrets can be reduced by the CB1-selective agonist methanandamide [128] but not by the CB2-selective agonists, JWH-133 or AM1241 [57].

Interestingly, when administered by itself SR141716A can induce vomiting in least shrews, albeit with a potency less than that with which it opposes the ability of cannabinoid receptor agonists to inhibit drug-induced vomiting [131]. There are also reports that AM251 can augment morphine-6-glucuronide-induced vomiting in ferrets [128] and that SR141716A-induced emesis in the least shrew can be blocked by CP55940, *R*-(+)-WIN55212, and Δ^9-THC, the rank order of the antiemetic potencies of these CB1/CB2 receptor agonists matching the rank order of their affinities for CB1 (and CB2) receptors [131].

As well as providing evidence that CB1 receptors can mediate inhibition of drug-induced vomiting, animal experiments have yielded data suggesting that these receptors mediate a nausea-suppressant effect. These data come from experiments in which rats were conditioned to display rejection reactions (gaping, chin rubbing and paw treading) to a saccharine solution as a result of prior paired exposure to saccharine and an emetic (cyclophosphamide or lithium chloride). These conditioned rejection reactions, which are thought to provide a measure of anticipatory nausea, were found to be suppressed by Δ^9-THC in cyclophosphamide-conditioned rats [132] and by Δ^9-THC and HU-210 in lithium chloride-conditioned rats [133]. The effect of HU-210 was attenuated by SR141716A. However, it is unclear whether SR141716A produced this antagonism by competing with HU-210 for CB1 receptors as when administered by itself, it increased the number of rejection reactions displayed by lithium chloride-conditioned rats.

Although it has been found that exogenous administration of 2-arachidonoyl glycerol reduces morphine-6-glucuronide-induced vomiting in ferrets [57], this endocannabinoid has also been reported to *induce* vomiting in the least shrew [134]. While the antiemetic effect of 2-arachidonoyl glycerol can be attenuated by AM251, its emetic effect is opposed both by cannabinoid-receptor agonists and by a subemetic dose of SR141716A. A further complication is that the antiemetic effect of 2-arachidonoyl glycerol in ferrets can also be attenuated by the CB2-selective antagonist, AM630. This finding, which contrasts with data obtained with anandamide and with other cannabinoid receptor agonists, raises the possibility that 2-arachidonoyl glycerol may produce its antiemetic effect in ferrets by activating both neuronal CB1 receptors and a population of CB2 receptors recently discovered to be present in ferret, rat, and mouse brainstem [57].

2.4 Loss of Appetite and Body Weight

Anecdotal reports that cannabis stimulates appetite, an effect that is now often referred to as “the munchies”, stretch back over many centuries [135]. This effect of cannabis, which has also been demonstrated in laboratory experiments

[136], is most likely CB1 receptor mediated. Thus, results from experiments with rats or mice indicate first that the ability of cannabis to stimulate food intake is shared by established CB1/CB2 receptor agonists such as Δ^9-THC, Δ^8-THC, *R*-(+)-WIN55212, anandamide, and 2-arachidonoyl glycerol and by the CB1-selective agonist, noladin ether, when these are administered peripherally or directly into areas of the brain involved in the control of feeding [137–149] (see also next paragraph) and second that the ability of some of these agonists to stimulate food intake in rats or mice is opposed by the CB1-selective antagonist, SR141716A, but not by the CB2-selective antagonist, SR144528 [143–148].

Results obtained from laboratory experiments also suggest first that cannabis and individual cannabinoids both increase the incentive to eat and render food more palatable and second that these effects are not limited to any particular flavors or food types or significantly affected by the context in which the food is presented [136, 150]. The hyperphagic effect of cannabinoid receptor agonists is thought to depend to a significant extent on the activation of CB1 receptors of the shell subregion of the nucleus accumbens. Thus, 2-arachidonoyl glycerol stimulates feeding in rats when injected directly into this brain area, and this effect is attenuated by SR141716A [144]. Moreover, dopaminergic neurons that project from the ventral tegmental area to the nucleus accumbens are thought to give rise to feelings of reward when activated [151], and there is evidence first that dopamine release from these mesolimbic neurons is increased not only by the process of eating and by electrical stimulation of the lateral hypothalamus but also by Δ^9-THC, and second that feeding in rats induced by electrical stimulation of the lateral hypothalamus is facilitated by Δ^9-THC [136, 151–153]. Cannabinoids may also stimulate feeding by interacting directly with the hypothalamus as, for example, experiments with rats have shown that anandamide elicits a hyperphagic response when injected into the ventromedial hypothalamus [143] and that Δ^9-THC increases food intake when injected into the paraventricular nucleus of the hypothalamus [145]. In addition, it is possible that cannabinoids induce hyperphagia by activating CB1 receptors expressed in hindbrain nuclei that are involved in the control of feeding and in vagal afferent neurons that project to these nuclei from the gastrointestinal tract [136, 154]. Interestingly, although it is well established that SR141716A decreases food intake when administered peripherally (Sect. 2.5) or intracerebroventicularly [145, 155], it has been reported that this CB1 receptor antagonist/inverse agonist does not suppress feeding in rats when injected into the paraventricular nucleus of the hypothalamus [145].

It has been demonstrated in several clinical trials that the hyperphagic effect of cannabinoids observed in healthy subjects extends to patients with cancer or human immunodeficiency virus (HIV) infection who exhibit excessive loss of appetite and body weight. In one of these studies, Regelson et al. [156] found that compared to placebo, daily oral administration of Δ^9-THC increased body weight in 16 patients with advanced cancer, most of whom were also receiving chemotherapy. In a later study, Plasse et al. [157] found Δ^9-THC (dronabinol) to increase appetite and/or reduce body weight loss both in cancer patients

(2.5 or 5 mg p.o. once or twice per day with or without concomitant chemotherapy) and in patients with symptomatic HIV infection (usually 2.5 mg p.o. up to three times per day). Struwe et al. [158] found dronabinol (5 mg p.o. twice per day) to increase body weight, in this case in a randomized, placebo-controlled, double-blind, crossover study performed with five HIV patients. Appetite improvement was also experienced by these patients in response to dronabinol. Similarly, Beal et al. [159] found that in a larger randomized, placebo-controlled, double-blind, parallel-group study, dronabinol (2.5 mg p.o. twice per day) increased appetite and stabilized body weight in patients with anorexia caused by acquired immunodeficiency syndrome (AIDS). Further evidence that dronabinol can stimulate food intake in HIV patients was obtained recently in a placebo-controlled study in which single, oral administration of this drug (10, 20, or 30 mg) was found to stimulate caloric intake in experienced cannabis smokers with HIV [160]. Interestingly, this stimulation was only observed in subjects with clinically significant muscle mass loss. It is likely that the dronabinol-induced decreases in body weight loss observed in many of these studies were mediated not only by CB1 receptors in the brain but also by peripheral non-neuronal CB1 receptors, there being evidence from experiments with mouse adipocytes [161] and hepatocytes [162] that CB1 receptors expressed by adipose tissue and the liver induce lipogenesis when activated.

It has been permissible since the 1980 s to prescribe dronabinol (Marinol®) in the United States to patients with AIDS who are experiencing loss of appetite and body weight. However, it still remains to be established whether CB1 receptor activation would also be an effective clinical strategy against these clinical signs when they are associated with other disorders such as anorexia nervosa or dementia or with ageing [136].

2.5 *Obesity*

The realization that CB1 receptor activation can stimulate food intake and increase body weight prompted two related hypotheses: (1) that the CB1 component of the endocannabinoid system has a physiological role in the regulation of appetite, eating behavior and body weight, and (2) that pathological overactivity of this system can lead to the development of obesity [136, 154, 163]. In line with the first of these hypotheses, it has been found that fasting increases 2-arachidonoyl glycerol and anandamide levels in rat limbic forebrain and 2-arachidonoyl glycerol levels in rat hypothalamus and also that the consumption of palatable food by nonfasted rats is associated before satiation with a reduction in hypothalamic 2-arachidonoyl glycerol levels [144]. Additional support for the hypothesis has come from experiments with free-feeding or food-deprived nonobese marmosets, rats, or mice showing that body weight and/or intake of normal or palatable food can be significantly reduced by the selective CB1 receptor antagonists, SR141716A, SR147778, AM251, or AM281, but not by the CB2-selective antagonists SR144528 or AM630 when

these are administered once (most experiments) or repeatedly [6, 146, 164–179]. It has also been found that repeated administration of AM251 can lead to a decrease in the feeding efficiency of nonobese rats, as measured by the ratio of change in body weight to total food intake [166]. Interestingly, although AM251 and SR141716A have the ability to reduce the food intake of nonobese rats at doses that these animals seem not to find aversive, higher doses of these antagonists do appear to be aversive, as indicated by results obtained in conditioned taste aversion experiments, suggesting that at relatively high doses these compounds may produce hypophagia at least in part by inducing effects such as emesis or malaise [166, 171, 180].

That SR141716A affects food intake and body weight by acting through CB1 receptors is supported by the findings, first that single administration of this antagonist does not decrease the food intake of nonobese $CB1^{-/-}$ mice [146, 169] and second that repeated administration of SR141716A does not reduce the food intake or body weight of $CB1^{-/-}$ mice with diet-induced obesity [181, 182]. Further support comes from reports that $CB1^{-/-}$ mice have lower body weight and/or adiposity than wild-type mice when fed a standard or obesity-inducing diet [161, 182, 183], and that $CB1^{-/-}$ mice consume less sucrose or less standard or high-fat diets than wild-type mice [161, 169, 182, 184]. It is noteworthy, however, that in the first-ever experiments performed with $CB1^{-/-}$ mice, the body weights of these animals were found to be no less than those of wild-type animals [185, 186].

The second hypothesis that overactivity of the endocannabinoid system can lead to the development of obesity is supported by both preclinical and clinical data. Turning first to the preclinical data, there are reports that SR141716A reduces the food intake of genetically obese *db/db ob/ob* and Lep^{ob}/Lep^{ob} mice and the body weights of obese *db/db* mice, Lep^{ob}/Lep^{ob} mice, and *Zucker (fa/fa)* rats when administered once or repeatedly [169, 178, 187, 188] and that single administration of AM251 reduces the food intake and overnight weight gain of obese *ob/ob* mice [189]. It has also been reported that single and repeated administration of SR141716A or AM251 reduces the food intake, body weight, adiposity/fat stores, and/or plasma free fatty-acid levels of mice with diet-induced obesity [167, 181, 190, 191]. It is likely that these effects of SR141716A and AM251 are due at least in part to an antagonism of CB1 receptor-mediated increases in feeding and body weight induced by endogenously released anandamide and/or 2-arachidonoyl glycerol. Thus, there are reports of increased hypothalamic levels of anandamide and 2-arachidonoyl glycerol in young obese *db/db* mice [169] and of 2-arachidonoyl glycerol (but not anandamide) in obese Zucker rats and young obese *ob/ob* mice [169]. Increased levels of 2-arachidonoyl glycerol and anandamide have been detected too in the uteri of *ob/ob* mice [192]. Since SR141716A and AM251 are CB1 receptor inverse agonists, it is also possible that they decrease feeding and/or body weight by reducing the constitutive activity of CB1 receptors. Such an action is expected to be amplified by CB1 receptor upregulation and it is noteworthy, therefore, that there are reports that CB1 mRNA expression is upregulated in the adipose

tissue of obese *fa/fa* rats [187] and that CB1 receptor density increases in mouse liver in response to a high-fat diet as measured by Western blotting [162]. These are both tissues in which CB1 receptors are thought to mediate lipogenesis (Sect. 2.4). It has also been found, however, that CB1 mRNA expression is down-regulated in the adipose tissue of obese women [193], and that body weight increases induced in rats by a palatable diet are associated with a decrease in the density of CB1-binding sites in extrahypothalamic brain areas, including the nucleus accumbens and hippocampus, which are involved in hedonic aspects of feeding [194].

Data obtained with SR141716A in experiments with unfasted nonobese rats [168] or with SR141716A or AM251 in experiments with diet-induced-obese mice [181, 190, 191] suggest that when these compounds are administered repeatedly it is possible for their attenuating effect on food intake to decline or disappear without any concomitant decrease in their body weight- or adiposity-reducing effects. These results are consistent with the hypothesis that CB1 antagonists can reduce body weight by antagonizing not only endocannabinoid-mediated hyperphagia but also endocannabinoid-mediated lipogenesis and fat accumulation [154]. However, it is noteworthy that Chambers et al. [166] have shown that it is possible to administer AM251 repeatedly to unfasted nonobese rats without rendering these animals tolerant to the hypophagic effect of this compound.

Obese animals appear to be more sensitive than lean animals to the hypophagic and body weight-reducing effects of CB1 receptor antagonists. Thus, it has been found that the inhibitory effect of chronic SR141716A on food intake and body weight is greater in obese *fa/fa* Zucker rats than in lean animals [178] and there are reports that single treatments with AM251 that reduce the food intake and body weight gain of obese *ob/ob* mice or diet-induced obese mice have little or no effect in lean animals [167, 189]. Not unexpectedly, the hypophagic effect of CB1 receptor antagonism appears to be reversible, as body weight gain and signs of rebound hyperphagia have been observed when SR141716A is withdrawn from obese *fa/fa* Zucker rats [178], when AM251 is withdrawn from diet-induced obese mice [190] and when SR141716A or AM251 is withdrawn from normal rats [166, 168] after a period of repeated administration.

Moving onto the clinical data that support the hypothesis that overactivity of the endocannabinoid system can lead to the development of obesity, there are reports first that SR141716A induces weight loss and a reduction both in waist circumference and in metabolic and cardiovascular disease risk factors in overweight or obese subjects when administered repeatedly [154, 195–197] and second that circulating levels of anandamide and arachidonoyl glycerol are significantly higher in obese than lean women [193]. It has also been reported that plasma levels of anandamide but not 2-arachidonoyl glycerol are elevated in obese women with binge-eating disorder, although not in female bulimia nervosa patients with body weight, body mass index, and body fat mass in the normal range [198]. The increased tissue levels of anandamide and/or arachidonoyl glycerol detected in obese women and animals may reflect increased

endocannabinoid synthesis and release, for example, in response to decreased tissue levels of leptin [136, 154, 169]. Another possible contributory factor is a reduction in the capacity to remove endocannabinoids from their sites of action, there being reports first that there is less fatty acid amide hydrolase (FAAH) mRNA in the adipose tissue of obese women than of lean women [193], second that human obesity and excessive body weight is associated with a missense polymorphism in the gene encoding FAAH [199], and third that both endocannabinoid cellular uptake and the activities of FAAH and monoacyl glycerol lipase are less in the uteri of *ob/ob* mice than of wild-type animals [192]. Further experiments will be required to establish the physiological consequences of the increased plasma levels of anandamide detected in obese women, not least because such increases have also been observed in underweight women with anorexia nervosa [198].

The results obtained with SR141716A (rimonabant; Acomplia®) in clinical trials have been most encouraging [154, 163, 197]. Thus, for example, in two 1-year clinical trials, conducted by Van Gaal et al. [195] and Després et al. [196] with overweight or obese subjects, rimonabant exhibited significant efficacy as an antiobesity agent and produced relatively minor adverse reactions in most patients, even at the highest test dose of 20 mg. Adverse reactions experienced by some patients in either or both of these trials included nausea, vomiting, diarrhea, headache, dizziness, arthralgia (pain in a joint), insomnia, influenza, feelings of anxiety, and depressed mood disorders. These occurred mainly during the first few months of rimonabant treatment. Because of these and other clinical findings, rimonabant has now been licensed in the UK as a prescription medicine for the management of obesity (since June 28, 2006). The extent to which a CB1 receptor antagonist like rimonabant might *exacerbate* unwanted symptoms in some patients is still to be fully investigated. These would be patients in whom endocannabinoid release onto CB1 receptors was providing some degree of protection from symptoms such as inflammatory pain or spasticity [3, 200] (Sect. 3). In the meantime, it is worth noting that when administered repeatedly, SR141716 has been reported to exhibit antinociceptive activity in a rat model of neuropathic pain [201].

3 Summary and Some Future Directions

This chapter has reviewed the evidence, published up to January 2006, that supports (1) the established therapeutic uses of cannabinoid receptor agonists as antiemetics, as appetite stimulants and as analgesics for the relief of neuropathic pain, (2) the potential therapeutic uses of cannabinoid receptor agonists for the amelioration of inflammatory or postoperative pain and of symptoms, including pain, associated with multiple sclerosis, and (3) the therapeutic use of CB1 receptor antagonists for the management of obesity. The only cannabinoid receptor ligands already licensed for use as medicines are the CB1/CB2 receptor agonist Δ^9-THC, its synthetic analog nabilone, the Δ^9-THC-containing,

cannabis-based medicine, Sativex®, and the CB1-selective antagonist/inverse agonist, Acomplia®. As to potential future developments, it is likely that a second generation of cannabinoid receptor agonists will eventually be developed for the clinic that, unlike Δ^9-THC or nabilone, target subpopulations of cannabinoid receptors and consequently possess greater selectivity. As discussed in greater detail elsewhere [3], these will most probably include CB2-selective agonists that exploit the ability of CB2 receptors to mediate relief from inflammatory and neuropathic pain (Sect. 2.1). They are also likely to include CB1 and/or CB2 receptor agonists that do not readily cross the blood–brain barrier and that exploit the ability of peripheral CB1 and CB2 receptors to mediate relief from inflammatory and neuropathic pain (Sect. 2.1).

In view of reports that pain relief can be mediated by cannabinoid receptors in the spinal cord and skin, it is also possible that the selectivity of a cannabinoid receptor agonist could be improved by administering it intrathecally or transdermally [3]. One other clinical strategy for improving selectivity could well be to coadminister a cannabinoid receptor agonist at a low dose with a noncannabinoid with which it interacts synergistically to produce its sought-after effect. It has been found that such synergism occurs between Δ^9-THC and opioid receptor agonists such as morphine and codeine for antinociception [202] and between Δ^9-THC and ondansetron for the suppression of vomiting and retching [203]. Interestingly, Δ^9-THC also appears to oppose the onset of tolerance to opioid-induced antinociception [202].

There is evidence that tissue concentrations of endocannabinoids increase in certain disorders and that these increases often lead to a reduction in the severity of symptoms or to a slowing of disease progression [3]. Such evidence has, for example, been obtained in experiments with animal models of multiple sclerosis and of inflammatory pain. This raises the possibility that cannabinoid receptor-mediated amelioration of multiple sclerosis or inflammatory pain might be achieved by augmenting this apparently protective upregulation of the endocannabinoid system with drugs that delay the removal of endocannabinoids from their sites of action or that allosterically enhance endocannabinoid-induced activation of cannabinoid receptors. Indeed, there have already been a number of reports that drugs that inhibit the cellular uptake and/or enzymic degradation of endocannabinoids are antinociceptive in animal models both of acute pain and of inflammatory pain and, also, that such drugs can reduce spasticity and signs of impairment of motor function in animal models of multiple sclerosis [3]. It is possible that a similar strategy could be employed for the management of nausea and vomiting as it was found recently by van Sickle et al. [57] first that morphine-6-glucuronide-induced vomiting in ferrets can be reduced by inhibitors of endocannabinoid cellular uptake (VDM11) or metabolism (URB 597) and second that levels of 2-arachidonoyl glycerol are elevated by VDM11 and levels of anandamide by URB 597 in the brainstems of anesthetized ferrets that have received intragastric hypertonic saline as an emetic stimulus. The ability of SR141716A or AM251 to induce vomiting in least shrews, to enhance drug-induced vomiting in ferrets, and to augment signs

of anticipatory nausea in lithium chloride-conditioned rats (Sect. 2.3) may be a further indication that there is an ongoing release of endocannabinoids onto CB1 receptors in these animal models and that this produces an attenuation of both nausea and vomiting. It is likely that for the management of disorders in which endocannabinoids are released primarily onto subpopulations of cannabinoid receptors that mediate symptom relief and/or slow disease progression, medicines that inhibit the uptake or metabolism of endocannabinoids, or that allosterically enhance their actions will exhibit greater selectivity than directly acting agonists that target all cannabinoid receptors following their exogenous administration.

Finally, there is evidence that the density of cannabinoid receptors sometimes increases in cells or tissues in which these receptors mediate symptom relief without increasing in all cannabinoid receptor-expressing tissues [3]. Such a pattern of receptor upregulation which has, for example, been observed in animal models of neuropathic pain is expected to improve the benefit-to-risk ratio of a cannabinoid receptor agonist by increasing the potency with which it produces its sought-after effects without affecting the potency with which it produces other effects. Upregulation of this kind may also increase the size of the maximal response to an agonist, particularly when this is a partial agonist such as Δ^9-THC, and so increase the maximal possible degree of agonist-induced symptom relief.

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